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DIVISION OF FISHES
U.S. NATIONAL MUSEUM

INVESTIGATIONS IN FISH CONTROL

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32. Toxicity of Hyamine 3500 to Fish
33. Voidance Time for 23 Species of Fish
34. Laboratory Studies on
Possible Fish-Collecting Aids
With Some Toxicities for the
Isomers of Cresol



United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

INVESTIGATIONS IN FISH CONTROL

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32. Toxicity of Hyamine 3500 to Fish

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TOXICITY OF HYAMINE 3500 TO FISH

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ABSTRACT.--Toxicity of Hyamine 3500 to three species of trout and 11 species of warmwater fish was determined in static bioassays. Twenty-nine lots of fish from nine sources were used in water at various levels of pH, temperature, and total hardness. Hyamine 3500 is more toxic in alkaline or acidic water than in water with a pH of 7. In general, toxicity varies directly with temperature and inversely with total hardness. Dilute solutions of Hyamine 3500 appear to degrade rapidly in open vessels.

Because of its efficacy as a microbicide and its relatively low toxicity to mammals (Rohm and Haas Co., 1965), Hyamine 3500 is being considered as a disinfectant in fish culture (Warren, 1964). Before it can be considered for general use, its toxicity to fish must be known. The purpose of this study was to determine the toxicity of Hyamine 3500 to several species of fish in waters of various qualities.

MATERIALS AND METHODS

The active ingredients in Hyamine 3500 are a selected blend of alkyl (C_{14} , 50 percent; C_{12} , 40 percent; and C_{16} , 10 percent) dimethyl benzyl ammonium chlorides having an average molecular weight of 358 g/mole. Two forms of Hyamine 3500 are available: an 80-percent concentrate in ethanol, and a 50-percent aqueous solution. We used the latter. The other ingredients in the aqueous solution include 40 percent of water and 10 percent of ethanol. This solution is pale yellow, has a mild odor, congeals at low temperatures, and is miscible in all proportions with water, lower alcohols, and ketones.

The species, lot numbers, weights, and sources of fish are listed in table 1. Most

of the lots were from National Fish Hatcheries, but two lots of goldfish were from a private producer, and one lot of channel catfish was from a Georgia State Fish Hatchery, and one lot of trout was from a Wisconsin State Fish Hatchery. The term "lot" refers to a particular group of fish of a species received from a hatchery in a single shipment.

Routine bioassays were conducted in accordance with the methods outlined by Lennon and Walker (1964), with slight modifications. In general, the formulation of water of various levels of total hardness, alkalinity, and pH was accomplished by the methods outlined by Marking (1966, 1967). Water temperatures in vessels were maintained at $12^{\circ} \pm 1^{\circ}$, $17^{\circ} \pm 2^{\circ}$, or $22^{\circ} \pm 1^{\circ}$ C.

Immediately before each bioassay, concentrated stock solutions of Hyamine 3500 were prepared with a deionized water-acetone (50/50, v/v) solvent. Concentrations were calculated on the basis of product rather than active ingredient. Aliquots of these stock solutions were measured directly into bioassay vessels to produce desired concentrations. The amount of acetone per bioassay vessel never was in excess of the volume tolerated by the test fish.

The data from each bioassay were analyzed by the method outlined by Litchfield and

Wilcoxon (1949). By this method, LC_{50} values (concentrations producing 50-percent mortality) and their 95-percent confidence intervals were calculated.

RESULTS

Trout.--The 24-hour LC_{50} values at 12° C. indicate that rainbow trout were less sensitive

to Hyamine 3500 than lake trout and brown trout (table 2). The 96-hour LC_{50} values for the three species ranged from 1.90 to 2.45 p p m ; rainbow trout were still the least sensitive. The 95-percent confidence intervals of lake trout and brown trout overlap, indicating no statistically significant differences in the sensitivity of these two species.

TABLE 1.--The 14 species of fish used in toxicity tests of Hyamine 3500

Species	Lot	Average weight and range (grams)	Source
Rainbow trout, <u>Salmo gairdneri</u>	368	0.8(0.5 - 1.1)	Manchester NFH, Ia.
Brown trout, <u>Salmo trutta</u>	309	1.2(0.7 - 1.9)	McNenny NFH, S. Dak.
Lake trout, <u>Salvelinus namaycush</u>	346	1.9(1.5 - 2.2)	St. Croix Falls SFH, Wis.
Goldfish, <u>Carassius auratus</u>	W107	1.3(1.1 - 2.2)	Welaka NFH, Fla.
Do.....	W116	1.3(0.8 - 2.1)	Tallassee, Ala.
Do.....	W133	1.4(1.0 - 1.8)	Do.
Do.....	W153	1.1(0.7 - 1.5)	Marion NFH, Ala.
Carp, <u>Cyprinus carpio</u>	W162	1.0(0.9 - 2.1)	Do.
Fathead minnow, <u>Pimephales promelas</u>	W110	0.7(0.6 - 1.0)	Do.
Do.....	W113	1.0(0.5 - 2.0)	Do.
Do.....	W124	0.8(0.6 - 1.4)	Do.
Do.....	W139	0.8(0.6 - 1.4)	Do.
Smallmouth buffalo, <u>Ictiobus bubalus</u>	W163	1.1(0.5 - 1.4)	Do.
Brown bullhead, <u>Ictalurus nebulosus</u>	W148	1.1(0.5 - 1.2)	Do.
Channel catfish, <u>Ictalurus punctatus</u>	W115B	2.2(1.6 - 3.3)	Cordele SFH, Ga.
Green sunfish, <u>Lepomis cyanellus</u>	W157	0.9(0.5 - 2.1)	Marion NFH, Ala.
Do.....	W164	0.9(0.6 - 1.1)	Do.
Bluegill, <u>Lepomis macrochirus</u>	W109	0.8(0.7 - 1.0)	Do.
Do.....	W111	1.1(0.7 - 1.4)	Do.
Do.....	W119	1.1(0.7 - 1.4)	Do.
Do.....	W128	1.2(0.8 - 1.6)	Do.
Redear sunfish, <u>Lepomis microlophus</u>	W112	1.4(1.1 - 2.4)	Do.
	W117	1.1(0.8 - 1.5)	Do.
Smallmouth bass, <u>Micropterus dolomieu</u>	W146	0.7(0.5 - 0.9)	Mammoth Springs NFH, Ark.
Largemouth bass, <u>Micropterus salmoides</u>	W140	0.8(0.6 - 1.2)	Welaka NFH, Fla.
Do.....	W141	0.6(0.4 - 0.8)	Warm Springs NFH, Ga.

TABLE 2.--Toxicity of Hyamine 3500 to three species of trout at 12° C. at 24, 48, and 96 hours of exposure

Species	Lot	LC_{50} and 95-percent confidence interval (ppm) at --		
		24 hours	48 hours	96 hours
Rainbow trout.....	368	3.65 (3.38 - 3.94)	2.80 (2.59 - 3.02)	2.45 (2.19 - 2.74)
Brown trout.....	309	2.61 (2.14 - 3.18)	2.13 (1.79 - 2.53)	1.95 (1.65 - 2.30)
Lake trout.....	346	2.74 (2.51 - 2.99)	2.35 (2.12 - 2.61)	1.90 (1.71 - 2.11)

Warmwater fish.--The 96-hour LC_{50} 's of Hyamine 3500 for 10 species of warmwater fish range from 0.32 to 2.25 p p m at 17° C. (table 3). The response of the fish varied somewhat for different exposure periods. For instance, 3-hour LC_{50} values show that small-mouth bass were the most sensitive, while 96-hour data indicate that bluegill were the most sensitive. Similarly, 3-hour LC_{50} 's show that brown bullhead were the most resistant, but 96-hour data indicate that green sunfish were the most resistant. Thus, some species having a relatively high resistance in short exposures may have a comparatively low resistance in longer exposures, and for other species the converse is true.

The toxicity varied among lots of a given species, and within the same lot. For example, the 96-hour LC_{50} of lot W111 of bluegills was twice that of lot W109. Two tests on goldfish from lot W116 gave 3-hour LC_{50} values of 6.55 and 10 p p m, but results generally were consistent and predictable.

Effects of temperature.--Usually, the toxicity of Hyamine 3500 increases with temperature as indicated by the LC_{50} values for lake trout (table 4), fathead minnow, and channel catfish (table 5). At 24 and 48 hours, the LC_{50} 's for lake trout at 17° are less than half those at 7° C. All of the 24-, 48-, and 96-hour LC_{50} 's for fathead minnow and channel catfish indicate increasing toxicity with an increase in temperature from 12° to 22° C. When both the 95-percent confidence interval and the LC_{50} value are examined, it is apparent that some of the differences in toxicity indicated by the LC_{50} 's alone are not statistically significant. Temperature changes appeared to have less effect on the toxicity of Hyamine 3500 to goldfish and bluegill than to the other two species tested. Some LC_{50} values indicate that toxicity increased with temperature, but other values show the opposite to be true. For instance, the 96-hour LC_{50} value for goldfish at 12° is 2.09 p p m and at 22° is 2.56 p p m, while the 3-hour LC_{50} at 12° is 13.30 p p m and at 22° it is 8.50.

Effects of water quality.--The toxicity of Hyamine 3500 to lake trout, fathead minnow, and bluegill is greater in soft water than in

hard water (tables 6 and 7). The 96-hour LC_{50} 's in hard water are almost twice those in soft water.

The toxicity to goldfish and fathead minnow is greater at both acidic and alkaline pH levels than at neutrality (table 8). The effect of pH on the toxicity of Hyamine 3500 does not appear to be as great as the effect of water hardness.

DISCUSSION

In general, for at least the initial 24 hours, the toxicity of Hyamine 3500 to the 14 species of fish was dependent on the concentration of chemical and duration of exposure. Toxicity increased only slightly when exposures were continued from 24 to 96 hours. For instance, green sunfish from lot W164 had 3- and 6-hour LC_{50} 's of 8.00 and 4.50 p p m, respectively, while the 24-, 48-, and 96-hour LC_{50} values were 2.32, 2.25, and 2.25 p p m, respectively. For many species the 24- and 48-hour LC_{50} 's or the 48- or 96-hour LC_{50} 's were identical, or when slight differences existed they were not statistically significant.

There are many possible causes for the comparatively slow increase in toxicity when exposures were continued from 24 to 96 hours. Marking (1966) indicated that this slowness may be caused by absorption and metabolism of the toxicant by the test fish, and by natural degradation of the chemical in solution. The latter would seem to be especially true of Hyamine 3500 since Nicholes and Burton (1961) found that solutions of Hyamine 3500 deteriorated when stored in open vessels. Furthermore, they indicated that both the chemically titratable activity and the germicidal efficiency of Hyamine 3500 solutions decreased upon standing. This deterioration was found to increase rapidly if the storage temperature was higher than 22° C.

Bioassays showed that not only fish from different sources but fish from the same source and even fish from the same lot differ in their sensitivity to Hyamine 3500. Marking (1966) found that geographic location, water quality, pond fertilization, herbicide application, and feeding and handling during rearing

TABLE 3.--Toxicity of Hyamine 3500 to 11 species of fish at 17° C. at 3 to 96 hours of exposure

Species	Lot	LC ₅₀ and 95-percent confidence interval (ppm) at --				
		3 hours	6 hours	24 hours	48 hours	96 hours
Goldfish.....	W107	--	--	2.71 (2.36 - 3.12)	2.18 (1.82 - 2.62)	2.18 (1.82 - 2.62)
Do.....	W116	10.00 (8.47 - 11.80)	--	--	--	--
Do.....	W116	6.55 (5.74 - 7.47)	--	--	--	--
Do.....	W116	--	3.24 (3.06 - 3.64)	--	--	--
Do.....	W133	--	--	1.60 (1.30 - 1.97)	1.60 (1.30 - 1.97)	1.49 (1.16 - 1.91)
Carp.....	W162	4.40 (3.93 - 4.93)	2.18 (1.93 - 2.46)	1.85 (1.74 - 2.07)	1.80 (1.64 - 1.98)	--
Fathead minnow.....	W110	--	--	1.11 (0.98 - 1.25)	1.06 (0.91 - 1.23)	0.98 (0.89 - 1.08)
Do.....	W124	3.70 (3.33 - 4.11)	2.50 (2.31 - 2.70)	--	--	--
Smallmouth buffalo....	W163	3.62 (3.26 - 4.02)	2.20 (2.00 - 2.42)	--	--	--
Brown bullhead.....	W148	10.60 (9.06 - 12.40)	6.20 (5.00 - 7.69)	2.13 (2.01 - 2.26)	2.15 (2.05 - 2.26)	1.59 (1.48 - 1.70)
Do.....	W148	--	--	2.80 (2.35 - 3.33)	--	--
Channel catfish.....	W115B	--	--	1.38 (1.27 - 1.50)	1.05 (0.95 - 1.16)	0.95 (0.83 - 1.09)
Green sunfish.....	W157	--	--	--	--	--
Do.....	W164	8.00 (6.78 - 9.44)	4.50 (3.95 - 5.13)	2.32 (2.19 - 2.46)	1.80 (1.59 - 2.04)	1.75 (1.47 - 2.08)
Bluegill.....	W109	--	--	0.95 (0.85 - 1.06)	2.25 (2.04 - 2.48)	2.25 (2.04 - 2.48)
Do.....	W111	3.59 (3.15 - 4.09)	--	0.72 (0.59 - 0.88)	0.72 (0.59 - 0.88)	0.64 (0.55 - 0.75)
Do.....	W128	--	1.81 (1.63 - 2.01)	--	--	--
Redear sunfish.....	W112	7.00 (3.91 - 12.53)	--	--	--	--
Do.....	W117	3.50 (2.92 - 4.20)	2.22 (1.82 - 2.71)	1.03 (0.86 - 1.22)	0.74 (0.58 - 0.95)	0.74 (0.56 - 0.98)
Smallmouth bass.....	W146	3.82 (3.54 - 4.12)	--	1.83 (1.73 - 1.94)	--	1.66 (1.54 - 1.79)
Do.....	W146	2.68 (2.50 - 2.87)	1.96 (1.85 - 2.08)	1.60 (1.51 - 1.70)	1.40 (1.35 - 1.46)	1.37 (1.29 - 1.45)
Largemouth bass.....	W140	--	--	1.52 (1.41 - 1.64)	1.21 (1.10 - 1.33)	1.13 (1.07 - 1.20)
Do.....	W141	2.95 (2.52 - 3.45)	3.15 (3.00 - 3.31)	--	--	--

TABLE 4.--Toxicity of Hyamine 3500 to lake trout at three temperatures at 24, 48, and 96 hours of exposure.

Temperature (°C.)	Lot	LC ₅₀ and 95-percent confidence interval (ppm) at --		
		24 hours	48 hours	96 hours
7	346	4.00 (3.31 - 4.84)	2.45 (2.15 - 2.79)	1.80 (1.58 - 2.05)
12	346	2.74 (2.51 - 2.99)	2.35 (2.12 - 2.61)	1.90 (1.71 - 2.11)
17	346	1.36 (0.91 - 2.04)	1.22 (0.86 - 1.73)	1.00 (0.74 - 1.36)

may influence the relative tolerance of specimens to a toxicant. Also, water quality and feeding and handling during the holding time immediately before testing may have a direct influence on the physiological condition of the test fish. The 24-, 48-, and 96-hour LC₅₀ values obtained with lots of goldfish from Welaka National Fish Hatchery and Tallassee, Ala., indicated that the fish had statistically significant differences in their tolerance to Hyamine 3500. The 24-hour LC₅₀ values from separate tests conducted on different occasions with brown bullheads from lot W148 and with small-mouth bass from lot W146 show that there were statistically significant differences in resistance among fish from the same lot.

It is interesting that the 24-hour LC₅₀ values did not vary over a wide range among the 14 species tested. The range for warmwater fishes was from 0.72 p p m for bluegill to 2.71 p p m for goldfish at 17° C. As a group the three species of trout were more resistant than warmwater fishes to Hyamine 3500 at 12° C. Of the four species of warmwater fish tested at 12° C., only goldfish were as resistant as the trout.

In the only field data currently available, Drake¹ reported on the use of Hyamine 3500 for control of gill disease at Pendills Creek National Fish Hatchery in Michigan. In these tests, lake trout held in lentic water were exposed to Hyamine 3500 for 1 hour and observed for 24 hours following exposure to determine mortality. The tests were conducted in two similar types of water having the following physical and chemical characteristics: (1) raceway water having temperatures ranging from 2° to 5° C., a pH of 7.2, and a total hardness of 51 p p m, and

(2) tap water with temperatures ranging from 6° to 10° C., a pH of 7.5, and a total hardness of 51 p p m. Under the conditions listed, a 1-hour exposure to 2 to 5 p p m of Hyamine 3500 killed all fish within 45 minutes following the test. Similar exposures to concentrations of 1.50 to 1.75 p p m killed half the fish, while exposure to concentrations ranging from 1.10 to 1.40 p p m resulted in 0- to 80-percent mortality. Drake stated that mortality was greater when water temperature exceeded 4° C. He concluded that Hyamine 3500 was not suitable for treatment of gill disease in lake trout at Pendills Creek National Fish Hatchery.

Considering Drake's report, and the fact that warmwater fish are as sensitive as trout to Hyamine 3500, it appears that more field data are required before any recommendations can be made regarding Hyamine 3500 for control of fish diseases.

CONCLUSIONS

1. Decreasing mortality among fish indicates rapid degradation of dilute solutions of Hyamine 3500 in open vessels.
2. Toxicity of Hyamine 3500 to fish decreases as water hardness increases.
3. In general, toxicity of Hyamine 3500 to fish decreases as water temperature decreases.
4. Hyamine 3500 is more toxic at acid and alkaline pH than at pH 7.
5. The three species of trout tested are as resistant to Hyamine 3500 as are warmwater fish.

¹ Letter from Peter G. Drake, Manager, Pendills Creek NFH, Brimley, Mich., 1966.

TABLE 5.--Toxicity of Hyamine 3500 to fish at three temperatures at 3 to 96 hours of exposure

Species	Lot	Temp. (°C.)	LD ₅₀ and 95-percent confidence interval (ppm) at --				
			3 - hours	6 - hours	24 - hours	48 - hours	96 - hours
Goldfish.....	W107	12	--	6.10 (5.45 - 6.83)	2.72 (2.32 - 3.18)	2.19 (1.90 - 2.52)	2.09 (1.82 - 2.40)
Do.....	W116	12	13.30 (12.43 - 14.23)	--	--	--	--
Do.....	W107	17	--	--	2.71 (2.36 - 3.12)	2.18 (1.82 - 2.62)	2.18 (1.82 - 2.62)
Do.....	W116	17	10.00 (8.47 - 11.80)	--	--	--	--
Do.....	W116	17	6.55 (5.74 - 7.47)	--	--	--	--
Do.....	W116	17	--	3.34 (3.06 - 3.64)	1.60 (1.30 - 1.97)	1.60 (1.30 - 1.97)	1.49 (1.16 - 1.91)
Do.....	W133	17	--	--	--	--	--
Do.....	W107	22	8.50 (6.97 - 10.39)	4.29 (3.97 - 4.63)	2.90 (2.64 - 3.19)	2.67 (2.43 - 2.98)	2.56 (2.24 - 2.92)
Fathead minnow.....	W110	12	7.59 (6.72 - 8.58)	4.00 (3.50 - 4.60)	1.53 (1.28 - 1.82)	1.18 (1.04 - 1.34)	1.13 (1.00 - 1.28)
Do.....	W113	12	--	--	--	--	--
Do.....	W110	17	3.70 (3.33 - 4.11)	2.50 (2.31 - 2.70)	1.11 (0.98 - 1.25)	1.06 (0.91 - 1.23)	0.98 (0.89 - 1.08)
Do.....	W124	17	--	--	--	--	--
Do.....	W110	22	2.75 (2.62 - 2.89)	--	0.86 (0.78 - 0.95)	0.86 (0.77 - 0.96)	0.83 (0.77 - 0.90)
Do.....	W113	22	3.20 (2.91 - 3.52)	--	--	--	--
Do.....	W118	22	2.53 (2.39 - 2.68)	1.42 (1.24 - 1.62)	--	--	--
Channel catfish....	W115B	12	--	--	1.80 (1.64 - 1.98)	1.40 (1.28 - 1.53)	0.98 (0.88 - 1.09)
Do.....	W115B	17	--	--	1.38 (1.27 - 1.50)	1.05 (0.95 - 1.16)	0.95 (0.83 - 1.09)
Do.....	W115B	22	--	--	1.01 (0.91 - 1.12)	0.87 (0.74 - 1.02)	--
Bluegill.....	W109	12	6.79 (5.56 - 8.28)	3.70 (3.27 - 4.18)	1.31 (1.11 - 1.54)	0.92 (0.83 - 1.02)	0.64 (0.54 - 0.76)
Do.....	W111	12	--	--	--	--	--
Do.....	W109	17	--	--	0.95 (0.85 - 1.06)	--	0.32 (0.22 - 0.47)
Do.....	W111	17	3.59 (3.15 - 4.09)	--	0.72 (0.59 - 0.88)	0.72 (0.59 - 0.88)	0.64 (0.55 - 0.75)
Do.....	W128	17	--	1.81 (1.63 - 2.01)	--	--	--
Do.....	W109	22	2.76 (2.58 - 2.95)	1.29 (1.11 - 1.50)	0.83 (0.75 - 0.92)	0.79 (0.71 - 0.88)	0.79 (0.71 - 0.88)
Do.....	W111	22	--	--	--	--	--

TABLE 6.--Toxicity of Hyamine 3500 to lake trout (lot 346) at various levels of total hardness at two temperatures

Temperature (°C.)	Total hardness as CaCO ₃ (ppm)	LC ₅₀ and 95-percent confidence interval (ppm) at --		
		24 hours	48 hours	96 hours
12.....	10	2.85 (2.57 - 3.16)	1.82 (1.69 - 1.92)	1.49 (1.37 - 1.62)
12.....	42	2.74 (2.51 - 2.99)	2.35 (2.12 - 2.61)	1.90 (1.71 - 2.11)
12.....	170	4.18 (3.67 - 4.77)	3.15 (2.89 - 3.43)	2.83 (2.53 - 3.17)
17.....	10	0.59 (0.43 - 0.81)	0.50 (0.36 - 0.69)	0.42 (0.31 - 0.59)
17.....	42	1.36 (0.91 - 2.04)	1.22 (0.86 - 1.73)	1.00 (0.74 - 1.36)
17.....	170	0.96 (0.74 - 1.25)	0.70 (0.59 - 0.84)	0.70 (0.59 - 0.84)

TABLE 7.--Toxicity of Hyamine 3500 to fish in waters of various qualities at 17° C.

Species	Lot	Average pH	Total hardness as CaCO ₃ (ppm)	96-hour LC ₅₀ and 95-percent confidence interval (ppm)
Fathead minnow.....	W113	6.99	11.0	0.82 (0.73 - 0.92)
Do.....	W113	7.54	41.3	1.29 (1.08 - 1.55)
Do.....	W113	8.15	150.0	1.67 (1.52 - 1.84)
Bluegill.....	W119	6.99	11.0	0.43 (0.33 - 0.56)
Do.....	W119	7.54	41.3	0.50 (0.38 - 0.65)
Do.....	W119	8.15	150.0	0.82 (0.65 - 1.03)

TABLE 8.--Toxicity of Hyamine 3500 to fish at three pH levels at 17° C.

Species	Lot	pH	LC ₅₀ and 95 percent confidence interval (ppm) at --		
			24 - hours	48 - hours	96 - hours
Goldfish.....	W153	5.0 ± 0.1	1.89 (1.60 - 2.23)	1.80 (1.56 - 2.07)	--
Do.....	W153	7.0 ± 0.1	2.17 (1.92 - 2.45)	2.00 (1.74 - 2.30)	1.83 (1.59 - 2.10)
Do.....	W153	9.0 ± 0.1	1.85 (1.62 - 2.11)	1.73 (1.57 - 1.90)	--
Fathead minnow	W139	5.0 ± 0.1	--	0.73 (0.64 - 0.84)	0.60 (0.49 - 0.74)
Do.....	W139	5.0 ± 0.1	0.74 (0.67 - 0.81)	0.62 (0.55 - 0.69)	0.37 (0.27 - 0.50)
Do.....	W139	7.0 ± 0.1	1.35 (1.24 - 1.47)	1.18 (1.07 - 1.30)	1.10 (1.00 - 1.21)
Do.....	W139	9.0 ± 0.1	1.04 (0.96 - 1.12)	0.93 (0.85 - 1.01)	0.93 (0.85 - 1.00)

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INVESTIGATIONS IN FISH CONTROL

33. Voidance Time for 23 Species of Fish

By Thomas H. Lane and Howard M. Jackson



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VOIDANCE TIME FOR 23 SPECIES OF FISH

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ABSTRACT.--Observations on 23 species of fingerling-size bioassay fish indicated that voidance time (time required for food residues to pass through the alimentary canal) ranged from 12 to 108 hours.

Many species of fish are used in bioassays of pesticides, pollutants, and other chemicals (Douglas, 1960; Ward and Irwin, 1961; and Gould and Irwin, 1965). Guidelines for the acquisition, acclimatization, and evaluation of bioassay fish were defined by Hart, Doudoroff, and Greenbank (1945) and Doudoroff et al. (1951). They recommended feeding specimens during the 10-day pretest holding period, but they advised that food be withheld for 1 to 2 days before bioassays and during bioassays. Lennon and Walker (1964) advocated withholding food from test fish for as long as 4 days before bioassay, depending on the life stage and species of fish. The objective of the withholding is to empty the digestive tract of food wastes before the fish is introduced into bioassay.

Ward and Irwin (1961) stated that fish in good condition may not be greatly affected by short periods without food, but they cited evidence that the resistance of fish to chemicals may decrease with longer periods of starvation. In any case, the period off feed should be held to a minimum. Accordingly, the purposes of this investigation were to measure voidance times for various freshwater fishes and to determine whether voidance time is constant for a given species at one or two temperatures.

Some information concerning digestion rates, food passage rates, nutrition, and feeding behavior of fish is scattered throughout the literature. Results are diverse for a

species, even though size of fish, water temperature, and other variables are similar. Many of the variables characteristically found in this type of investigation are either referred to or briefly discussed by Darnell and Meierotto (1962) in their study on digestion rate in a population of small black bullheads. According to these authors, digestion rate and rate of food passage through the digestive tracts of fishes have been shown to vary with temperature, age of experimental animal, and type and amount of food. They also point out that digestion rates are affected by the general activity of the animals which in turn may be influenced by the light regimen.

Phillips et al. (1960) in a test using brook trout averaging 6 grams observed no significant change in rate of food passage which could be ascribed to a temperature reduction of 2.8° C., from 11.1° to 8.3°. Earlier studies showed a deceleration in food passage in fingerling brook trout when the temperature was reduced 8.3° C., from 10.5° to 2.2° (Phillips et al., 1956). Markus (1932) and Baldwin (1957) demonstrated in experiments with warm- and cold-water fish that a temperature change of 4° C. can appreciably affect digestion rate.

Phillips et al. (1956) observed that 6-gram brook trout showed a significant change in food passage rate when fed different types of food, that is, meat and a mixture of meat and dry meal.

METHODS AND MATERIALS

The observations on voidance time were made at the Fish Control Laboratory at La Crosse, Wis. and the Southeastern Fish Control Laboratory at Warm Springs, Ga. The water in the indoor holding facilities at La Crosse is supplied from a deep well. It is hard (220 to 330 parts per million as CaCO_3), and its temperature during the test was 12.0 ± 1.0 C. The holding and test temperatures were so similar that no acclimation was necessary when transferring fish into test facilities.

The water at Warm Springs for outdoor holding facilities and indoor testing is supplied by a spring and is hardened with lime to 35 to 45 ppm as CaCO_3 . Its temperature is 17.0 C. Test temperatures ranged from 12.0 to 25.0 , and experimental fish were carefully acclimated to them.

Most of the fish were acquired from Federal and State hatcheries. Golden shiners and brown bullheads were obtained from private ponds. The list of species is given in table 1.

The fish were fed routinely during the holding period, with special care taken within the last 24 hours before trials to provide as

TABLE 1.--Fish used in voidance tests

Rainbow trout, <u>Salmo gairdneri</u> .
Lake trout, <u>Salvelinus namaycush</u> .
Northern pike, <u>Esox lucius</u> .
Goldfish, <u>Carassius auratus</u> .
Carp, <u>Cyprinus carpio</u> .
Golden shiner, <u>Notemigonus crysoleucas</u> .
Fathead minnow, <u>Pimephales promelas</u> .
White sucker, <u>Catostomus commersoni</u> .
Bigmouth buffalo, <u>Ictiobus cyprinellus</u> .
White catfish, <u>Ictalurus catus</u> .
Black bullhead, <u>Ictalurus melas</u> .
Yellow bullhead, <u>Ictalurus natalis</u> .
Brown bullhead, <u>Ictalurus nebulosus</u> .
Channel catfish, <u>Ictalurus punctatus</u> .
Green sunfish, <u>Lepomis cyanellus</u> .
Pumpkinseed, <u>Lepomis gibbosus</u> .
Bluegill, <u>Lepomis macrochirus</u> .
Longear sunfish, <u>Lepomis megalotis</u> .
Smallmouth bass, <u>Micropterus dolomieu</u> .
Largemouth bass, <u>Micropterus salmoides</u> .
White crappie, <u>Pomoxis annularis</u> .
Yellow perch, <u>Perca flavescens</u> .
Walleye, <u>Stizostedion v. vitreum</u> .

much food as the fish would eat. Carnivorous species were furnished with live foods such as daphnia and fish fry. Some lots of fish were supplied with dry food, but most lots were given a synthetic food originally formulated at the Southeastern Fish Cultural Laboratory, Marion, Ala., for use in studies on the nutrition of channel catfish (Harry K. Dupree and Kermit E. Sneed. Purified diets for channel catfish nutritional research. Manuscript.)

All voidance observations at La Crosse and initial tests at Warm Springs were conducted in 1-gallon wide-mouth jars. Later experiments were accomplished in funnel-type aquariums which proved more useful (fig. 1). Cover screens were necessary on the funnel aquariums to prevent escape of some species and to minimize the effect of the observer's movements. Bottom screens of epoxy-coated hardware cloth were installed to prevent coprophagy.

Each test vessel in a battery of four vessels per species contained 2.5 liters of reconstituted, deionized water which was continuously aerated (Lennon and Walker, 1964). The fish were loaded into vessels at a rate of about 8 grams per liter of water. Observations were made twice daily on the fish and feces; any dead fish were removed; and the presence or absence of fecal material was noted. At each observation, test vessels containing significant amounts of solid feces were scored with a plus (+). Those containing only one or two small deposits or none were scored with a minus (-).

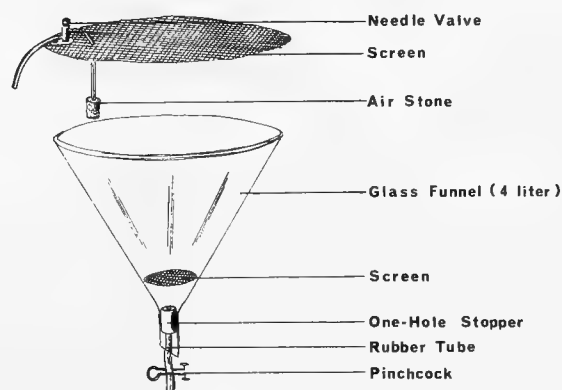


Figure 1.--Funnel-type aquarium used in voidance tests.

The tests of most species continued for 4 or 5 days. Voiding was judged to be complete at the end of the first interval during which two of the four test vessels were free of fecal deposits, that is, when a 50-percent reduction of + observations occurred. If three vessels were free of feces at the end of a period, voidance was listed as complete at the previous observation period, 12 hours earlier.

At La Crosse, the fish were transferred to clean jars at each observation period. At Warm Springs, about 10 percent of the water was removed from each jar each 12 hours and replaced with fresh reconstituted water.

Three techniques were used in identifying and enumerating feces: (1) Discrete fecal deposits were observed and counted; (2) Sudan 111, a red dye insoluble in water but soluble in oil, was added to the Marion catfish diet at 1 part per thousand to permit differentiation of food-waste feces from non-food feces which were found after last meals were completely voided; (3) A colorimetric method of detecting Sudan 111 in food-waste feces was employed.

The colorimetric method involved placing fecal samples in a Squibb separatory funnel after excess water was decanted. Glass beads and 20 milliliters of chloroform were added to the funnel and shaken vigorously until the dye was extracted. The bottom layer of chloroform with dye was drawn off and read in a DB spectrophotometer at a wavelength of 510 m μ , as percent transmittance. The peak absorbance of this wavelength was determined previously for Sudan 111 which was extracted from fecal material with chloroform. Readouts for each of four replications were averaged and plotted for each observation period (fig. 2).

During preliminary colorimetric experiments, reference blanks of solvent-extracted feces from fish fed the same synthetic diet without dye and blanks of 100-percent solvent were compared. The transmittance of the blanks was not significantly different, and the chloroform blank was used, therefore, in tests plotted in figure 2.

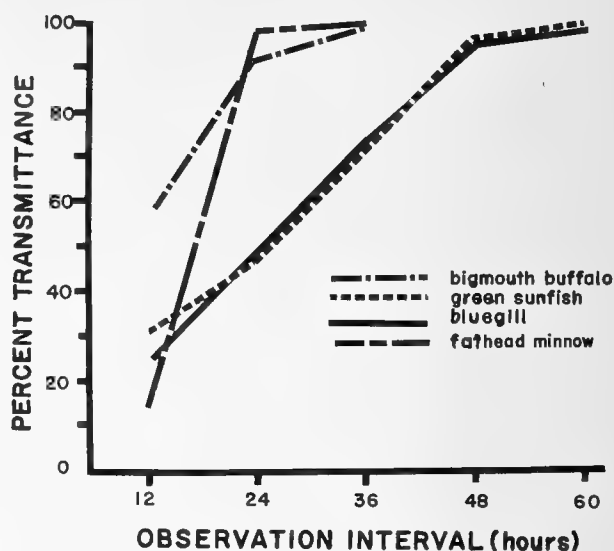


Figure 2.--Relative concentrations of Sudan 111 in fecal voidings of four fishes.

RESULTS

At La Crosse, 30 of 32 groups of fish voided within 36 to 96 hours at 11 $^{\circ}$ to 13 $^{\circ}$ C. (table 2). Metabolism is lower at these temperatures, and fish generally require more time to complete voiding. One group of lake trout did not complete voiding for 108 hours. One lot of channel catfish voided in less than 36 hours but it had appeared to feed poorly during pre-trial holding. Another lot of channel catfish, a lot of yellow perch, and one of bluegill fed only lightly during holding, and the intestines of several specimens from each lot were examined during the tests. Because of the comparatively small amounts of food found in them, the voiding results probably should be regarded as minimal values (table 2).

In tests at Warm Springs in warm water (19 $^{\circ}$ to 25 $^{\circ}$), 13 of 17 groups of fish completed voiding within 36 to 60 hours (tables 3 and 4). One group each of goldfish and yellow bullheads required 72 hours to complete voiding, and one group each of fathead minnows and bigmouth buffalo completed voiding in less than 36 hours.

Three groups of bluegills from lot number 17 (table 4) were tested at 12 $^{\circ}$, 17 $^{\circ}$, and 22 $^{\circ}$ C. to ascertain the effects of temperature on voiding. The voidance times were 84, 48, and 36 hours, respectively. The expected

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TABLE 2.--Voidance time for fish held at 12⁰+1⁰ C. at La Crosse, Wisconsin

Species	Total length (inches)		Weight (grams)		Number of fish	Number of replicates	Time to void (hours)
	Average	Range	Average	Range			
Rainbow trout.....	1.0	--	0.2	--	90	3	36
Do.....	--	1.6-2.4	1.2	--	30	3	60
Do.....	2.8	2.5-2.9	3.1	--	24	4	60
Lake trout.....	2.8	2.4-3.2	2.5	--	32	4	108
Do.....	3.7	3.4-4.1	5.6	4.4-6.5	12	4	60
Northern pike.....	1.5	1.3-1.7	0.4	0.2-0.8	40	4	¹ 72
Do.....	2.9	2.6-3.1	1.9	--	80	8	72
Goldfish.....	1.4	1.2-1.9	0.8	0.6-1.5	100	4	48
Do.....	1.8	1.5-2.7	2.5	--	74	7	36
Carp.....	2.0	1.6-2.5	2.5	2.0-3.6	80	8	60
Fathead minnow....	2.1	1.7-2.5	1.7	0.9-3.1	48	4	36
White sucker.....	--	3.5-3.9	6.0	--	12	3	60
Black bullhead....	2.2	1.6-2.5	2.1	0.9-3.2	36	4	84
Channel catfish....	--	--	2.1	1.4-3.7	36	4	² 24
Do.....	3.5	3.0-4.1	5.2	3.5-8.0	16	4	² 36
Green sunfish.....	1.6	1.3-2.0	1.4	0.5-1.7	72	4	60
Pumpkinseed.....	--	1.7-2.5	2.6	--	45	3	84
Bluegill.....	1.7	1.5-2.1	1.1	0.8-2.1	80	4	² 60
Do.....	--	1.2-2.0	1.2	--	60	3	84
Do.....	1.8	1.4-2.0	1.3	0.8-2.0	60	4	36
Longear sunfish....	1.6	1.4-2.0	1.0	0.7-1.8	125	7	72
Smallmouth bass....	1.3	1.1-1.6	0.5	0.3-0.7	40	4	¹ 72
Do.....	1.9	1.5-2.1	1.3	--	120	8	48
Largemouth bass....	1.4	1.2-1.4	0.5	--	160	4	84
Do.....	1.8	1.4-2.0	0.8	--	96	4	48
Yellow perch.....	1.4	1.2-1.7	0.5	--	160	4	60
Do.....	1.9	1.8-2.2	0.8	0.9-1.1	100	4	² 36
Walleye.....	1.6	1.2-2.0	0.5	--	360	12	60

¹ Observations made at 24-hour intervals.² Fish may not have consumed sufficient food before being placed in test vessels.TABLE 3.--Voidance time for fish held at 20⁰+1⁰ C. at Warm Springs, Ga.

Species	Weight (grams)		Number of fish	Number of replicates	Time to void (hours)
	Average	Range			
Goldfish.....	1.5	0.5-4.9	80	8	72
Do.....	1.2	0.9-2.0	40	4	60
Golden shiner.....	1.4	0.5-3.5	40	4	36
Fathead minnow....	0.9	0.6-1.9	40	4	12 ¹ (24)
Bigmouth buffalo...	1.5	1.2-3.0	40	4	24 ¹ (24)
Do.....	1.5	1.2-3.0	40	4	48
White catfish.....	3.2	2.0-5.1	40	4	48
Brown bullhead....	0.6	0.6-0.9	40	4	60
Green sunfish.....	1.2	0.7-2.0	40	4	48 ¹ (48)
Bluegill.....	1.2	0.4-2.0	40	4	36 ¹ (48)
Do.....	1.6	0.9-2.0	40	4	48
Do.....	1.6	0.6-4.0	80	8	60
Largemouth bass....	1.2	0.5-2.0	40	4	60
White crappie.....	0.4	0.2-0.7	40	4	60

TABLE 4.--Voidance time for fish held at various temperatures at Warm Springs, Ga.

Species	Lot number	Weight (grams)		Number of fish	Number of replicates	Average temp. (°C.)	Time to void (hours)
		Average	Range				
Goldfish.....	13	0.8	0.6-0.9	40	4	25.0	60
Carp.....	14	3.0	1.8-4.2	40	4	23.5	48
Do.....	14	3.6	2.4-5.6	40	4	22.5	48
Yellow bullhead	15	1.5	0.8-2.0	40	4	24.0	72
Bluegill.....	16	0.5	0.1-0.9	40	4	25.0	36
Do.....	17	0.6	0.4-1.5	20	2	22.0	36
Do.....	17	0.6	0.4-1.5	20	2	17.0	48
Do.....	17	0.6	0.4-1.5	20	2	12.0	84

¹ Values obtained by colorimetric method.

physiological effects of increased temperature were apparent, especially in tests made at 12° and 17°.

Visual assessments of voidance time for four species were made concurrently on samples which were evaluated by using the transmittance-time curve of the colorimetric method (fig. 2), and they were well corroborated by the latter.

DISCUSSION

The 1-gallon test jars used at La Crosse and initially at Warm Springs had several disadvantages. They made it necessary to handle the fish at each 12-hour period, which resulted in unnecessary stress and stress-induced defecation. Several types of test vessels were tried, but those which were funnel-like in design proved to be the most satisfactory. The design shown in figure 1 minimized handling of fish and permitted easy and efficient recovery of solid wastes which were concentrated in the neck of the funnel. According to Brockway (1950) excretory products in solution also tend to accumulate on the bottom in ponds and raceways because of their greater density. We presumed, therefore, that a large portion of liquid wastes at the bottom of an aquarium would be drawn off with the 250-milliliter sample of excrement and water.

The later feeding with the synthetic diet eliminated the early variance due to different types of foods. Some of the early variations in results with a species tested both at La Crosse and Warm Springs were due more to different types of food than to different temperatures. The synthetic diet also facilitated the use of a dye, Sudan 111, to detect the end point of voidance. It was acceptable, either with or without the dye, to all of the species tested except largemouth bass. Some species preferred it over several types of dry food. The gastric and intestinal mucosae of fish which were fasted for several days after eating dyed food were examined for evidence of dye absorption, but none was found.

The frequency of taking food varied with each test lot so that no pattern could be followed in feeding the fish. Food was offered frequently for 24 or more hours prior to

testing, and each time to the point of satiation.

The fish used in the voiding experiments were exposed to a change in water hardness. The reconstituted deionized water has a calcium hardness of about 16 ppm as CaCO_3 . Holding water at La Crosse has a calcium hardness range of 150 to 232 ppm, and that at Warm Springs ranges from 35 to 45 ppm. Phillips et al. (1954) observed that a diminution in water calcium was accompanied by an increase in metabolic activity in brook trout. A variable such as this may have influenced voidance times for fish at both laboratories.

Of the factors shown to affect digestion rates and food passage rates in fish (Darnell and Meierotto, 1962), temperature and those factors associated with diets and feeding probably are the most important contributors to the variance in voidance times. Large differences were found between voidance times of fish of the same species and size from different hatchery lots which were tested at the same temperature. This observation leads us to believe that interpopulation differences may be significant in causing variation in voidance time. The test results for different lots of fish of the same species and size were not averaged so that the differences would remain apparent. Even so, sufficient data are not available to evaluate effects which might result from population differences, and the question remains unanswered.

The problem of distinguishing between fecal deposits which contain food residues and those which do not became evident in early experiments with green sunfish, carp, goldfish, and bigmouth buffalo. According to Hawk, Oser, and Summerson (1954), feces resulting from food digestion and absorption are composed of (1) food residues, (2) the remains of intestinal and digestive secretions, (3) substances secreted into the intestinal tract, principally salts, (4) bacterial flora and their metabolic end products, (5) cellular elements, and (6) abnormal elements. During fasting, some species apparently void large quantities of any one, or possibly all, of the elements mentioned above except food residues. In one test not included in the tables, bigmouth buffalo voided for 156 hours without any noticeable decrease in the quantity or change in the

appearance of the fecal deposits. However, two tests with dyed food were performed with fish from the same lot. The results showed that voidance of food residues was complete in 24 and 48 hours (table 3) even though deposition of fecal material continued as before. Thus, the value of the dye method is clearly demonstrated.

The colorimetric method, although time-consuming, was used as a check on the visual method. It is considered more accurate since all of the excreted dye can be extracted and measured.

CONCLUSIONS

1. All but 2 of the 32 groups of fish tested at La Crosse at 11° to 13° C. completed voiding in 36 to 96 hours. One group of lake trout required 108 hours, and one group of channel catfish, which fed poorly, completed voiding in less than 36 hours.
2. All but 4 of 17 groups of fish tested at Warm Springs at 19° to 25° C. completed voiding in periods of 36 to 60 hours. One group each of goldfish and yellow bullheads required 72 hours, and one group each of fathead minnows and bigmouth buffalo completed voiding in less than 36 hours.
3. Solid feces formed as a result of feeding and those formed during fasting could not be discriminated in green sunfish, carp, goldfish, and bigmouth buffalo without use of a dye marker. Voidance times determined using the dye, Sudan 111, were corroborated by the colorimetric method.
4. The period of time that fingerling-size fish should be held off feed before use in bioassays depends principally on the species, type of food, and temperature. If contamination of bioassays by food-waste feces is to be minimized, food should be withheld from most of the 23 species studied for 2 to 3 days before bioassays.

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34. Laboratory Studies on Possible Fish-Collecting Aids With Some Toxicities for the Isomers of Cresol

By Robert M. Howland



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LABORATORY STUDIES ON POSSIBLE FISH-COLLECTING AIDS, WITH SOME TOXICITIES FOR THE ISOMERS OF CRESOL

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ABSTRACT.--The relative merits of quinaldine (2 methylquinoline), McNeil-JR-7464 (dl-1-(1-phenyl-ethyl)-5-(propoxy-carbonyl)-imidazole hydrochloride), and three isomers of cresol (p-methylphenol, o-methylphenol, and m-methylphenol) as collecting agents were determined in a lotic system under laboratory conditions at 12°C. The toxicity of the three cresol isomers to rainbow trout, brown trout, and brook trout also was measured in bioassays conducted in standard constituted water, and LC50 values were calculated for exposures of 6, 24, 48, and 96 hours. The toxicity of para-cresol was also established for carp, fathead minnow, black bullhead, channel catfish, bluegill, and yellow perch. Conclusions of these toxicity and efficacy tests were as follows: (1) Para-cresol is the most active of the three isomers of cresol in inducing total incapacitation of rainbow, brown, and brook trout; (2) The cresols are too harsh physiologically to warrant further development as fish-collecting agents; (3) MS-222 is not well suited as a fish-collecting tool; it does not induce surfacing and a high concentration is necessary for total incapacitation; (4) Quinaldine may have some usefulness for collecting fish in small streams with rapid flow but its physiological effects need elucidation; (5) McNeil-JR-7464 does not induce surfacing but is effective at low concentrations and appears to have desirable characteristics as a fish-collecting agent which merits further investigation.

Chemical compounds are needed for collecting fish without harming them. Such compounds as rotenone, cresol, quinaldine, aqualin, and tranquilizers have come under scrutiny (Wilkins, 1955; Louder and McCoy, 1962; Blanchard, 1965; Penfold, 1965; Tate, Moen, and Severson, 1965). Chemically induced surfacing of fish has been considered a desirable characteristic by some investigators (Loeb, 1962; Blanchard, 1965). Commercial cresol evoked a degree of surfacing, according to Wilkins (1955), when used as a stream sampling aid by the Tennessee Game and Fish Commission.

Because of the performance of cresol in past trials (Embody, 1940; Wilkins, 1955), we decided on further laboratory studies on the chemical. We used static bioassay to determine the toxicities of three isomers of cresol to rainbow trout, brown trout, and brook trout. The toxicity of para-cresol was also determined on carp, fathead minnows, black bullheads, channel catfish, bluegill sunfish, and yellow perch. The efficacies of ortho-cresol, meta-cresol, and para-cresol as collecting aids were measured with rainbow trout. Subsequently, quinaldine, MS-222, and McNeil-JR-7464 were investigated along with the

most effective isomer of cresol, against rainbow trout, to compare their relative merits as collecting agents in a lotic system.

METHODS AND MATERIALS

Chemicals

The isomers of cresol were a practical grade of para-cresol (p-methylphenol) and a purified grade of ortho-cresol (o-methylphenol) and meta-cresol (m-methylphenol). The quinaldine (2-methylquinoline) was practical grade. The McNeil-JR-7464 is dl-1-(1-phenyl-ethyl)-5-(propoxy-carbonyl)-imidazole hydrochloride.

Toxicity

Static bioassays with the isomers of cresol were with acclimated 2-inch fish in 5-gallon glass jars (Lennon and Walker, 1964). We exposed 10 fish to each concentration, and 20 served as controls. The statistical methods of Litchfield and Wilcoxon (1949) were used for determining LC50 values, slope functions, and 95-percent confidence intervals. Observations were made on surfacing and signs of physiological stress.

Efficacy

The lotic system for constant-flow experimentation was a modified aluminum hatchery through 14 feet long (fig.1). A head-box

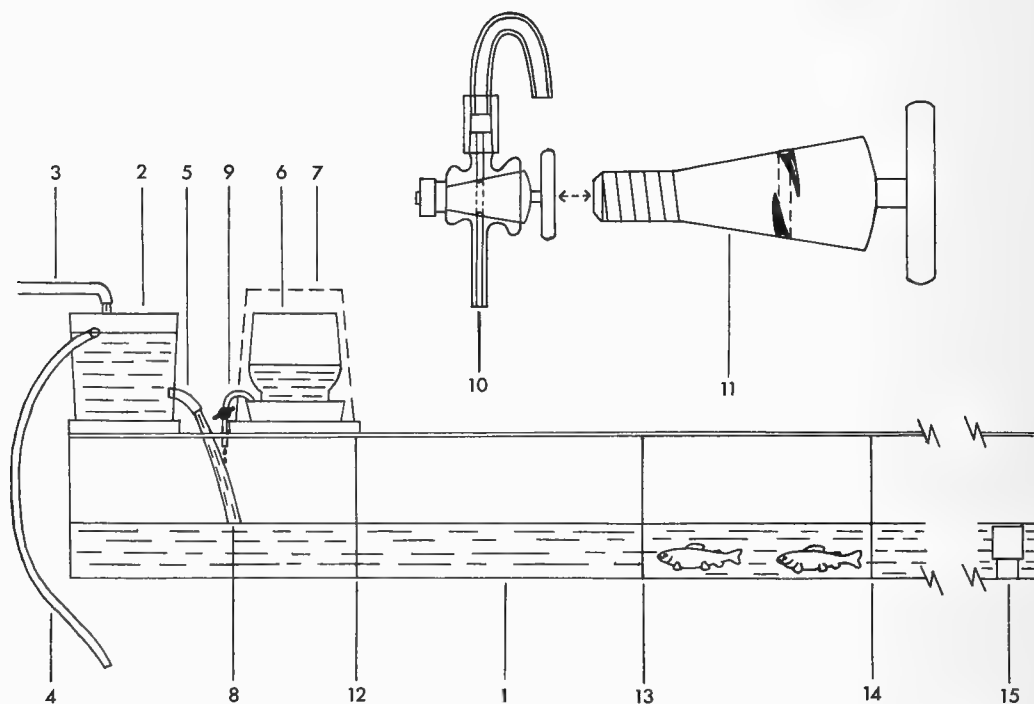


Figure 1.—A lotic system for constant-flow experimentation: (1) standard aluminum hatchery trough, (2) head-box, (3) water supply, (4) overflow tube, (5) spout, (6) 1-gallon poultry waterer, (7) inverted-plastic-pail cover to reduce evaporation and noxious fumes, (8) admixture of inflowing water and chemical solution, (9) siphon tube, (10) enlargement of siphon tube, (11) modified teflon stopcock, notched at each hole with a shallow tapering v-groove to increase flow adjustability, (12) diffusion screen, (13 and 14) test chamber screens, (15) outlet standpipe with adjustable rubber sleeve for depth regulation.

provided a uniform flow of water. Chemical-stock solution was held in a 1-gallon poultry waterer positioned below the head-box to allow admixture of chemical and inflowing water. Metering-in of the stock solution was accomplished with a glass siphon tube, equipped with an adjustable stopcock, modified from a standard burette. With this arrangement we could maintain a flow of 20 milliliters per minute with solutions of different viscosities, for the short periods involved in the experiments.

The trough was divided into compartments with screens 1 foot, 3.5 feet, and 5.5 feet from the point of inflow. The first screen served to enhance mixing and the following screens formed a test compartment for the fish. The inflow from the head-box was adjusted by using spouts of different diameters. The required amounts of chemicals and stock solutions were determined by the method described by Burrows (1949).

The stocks for quinaldine and the cresols were prepared with a 50:50 acetone:water solution. MS-222 and McNeil-JR-7464 dissolved readily in water. The progress of chemicals through the trough was simulated with 5 ppm (parts per million) of malachite green. Samples taken 1 minute apart at the head-screen of the fish compartment and analyzed by spectrophotometer provided a chronology of the increase toward maximum concentration. Thus, parameters were established for the subsequent exposure of test fish.

To appraise the value of these chemicals for collecting purposes, criteria of response were established in preliminary trials with cresol (mixture) and 3-inch rainbow trout:

1. Repellency minimal to reduce evasion in areas of weak concentration; surfacing desirable.
2. Total incapacitation, i.e. loss of equilibrium with only weak fin and directional movements remaining, within 10 minutes of contact.
3. Recovery not so rapid as to interfere with collecting.

4. No sign of physiological damage. We used twenty 3-inch rainbow trout to test each concentration for determining the most active isomer of cresol. Ten 9-inch rainbow trout were used at each concentration in the comparative testing of four compounds. Fish used in the lotic system were acclimated overnight. We kept a chronological record of fish responses for each trial. The flow of chemical was halted when all fish were in total incapacitation or when this stage was not attained within 15 minutes.

All tests were carried out at 12°C. in re-constituted water (Lennon and Walker, 1964). Table 1 lists the sources and sizes for test fish.

TABLE 1.--Species, sizes, and sources of test fish

Species	Average length (inches)	Source
Rainbow trout, <i>Salmo gairdneri</i>	2	Nevin, Wis., SFH. ¹ Manchester, Iowa, NFW. ²
Do.....	3	Troutlodge Springs, Soap Lake, Wash.
Do.....	9	Manchester, Iowa, NFW.
Brown trout, <i>Salmo trutta</i>	2	Do.
Brook trout, <i>Salvelinus fontinalis</i> ..	2	Osceola, Wis., SFH.
Carp, <i>Cyprinus carpio</i>	2	Lake Mills, Wis., NFW.
Fathead minnow, <i>Pimephales promelas</i> .	2	Bud's Marina, La Crosse, Wis.; collected from Minnesota lakes
Black bullhead, <i>Ictalurus melas</i>	2	Necedah, Wis., NWR. ³
Channel catfish, <i>Ictalurus punctatus</i>	2	Fairport, Iowa, NFW.
Bluegill, <i>Lepomis macrochirus</i>	2	Lake Mills, Wis., NFW.
Yellow perch, <i>Perca flavescens</i>	2	Do.

¹ State Fish Hatchery.

² National Fish Hatchery.

³ National Wildlife Refuge.

RESULTS

Toxicity

The three isomers of cresol were toxic to trout (table 2). The 24-hour LC50's reveal the differences in toxicity most clearly: against rainbow trout the value for para-cresol was 9.2 ppm, for ortho-cresol 9.9 ppm, and for meta-cresol 10.4 ppm. Similarly, against brown trout, para-cresol produced a value of 4.4 ppm, ortho-cresol 7.2 ppm, and meta-cresol 8.6 ppm. The values against brook

TABLE 2.--Toxicities of the isomers of cresol to fish at 12° C.

Isomer	Species of fish	LC50 (ppm) and 95-percent confidence interval at				Mean slope function
		6 hours	24 hours	48 hours	96 hours	
<u>ortho</u> -cresol.....	Rainbow trout	11.0 9.6-12.7	9.9 8.8-11.1	8.6 7.6-9.8	7.0 6.1-8.0	1.400
Do.....	Brown trout	--	7.2 6.7-7.8	7.2 6.7-7.8	6.2 5.2-7.4	1.253
Do.....	Brook trout	14.1 10.4-19.0	7.9 5.3-11.8	7.8 5.9-10.3	7.2 6.7-7.7	2.135
<u>meta</u> -cresol.....	Rainbow trout	14.9 13.4-16.6	10.4 9.3-11.6	10.2 9.3-11.2	8.6 7.7-9.6	1.228
Do.....	Brown trout	11.0 9.7-10.6	8.6 7.5-9.8	8.4 7.4-9.6	8.4 7.8-9.6	1.288
Do.....	Brook trout	11.4 9.7-13.4	8.2 7.7-8.8	7.6 7.0-8.2	7.6 7.0-8.2	1.208
<u>para</u> -cresol.....	Rainbow trout	11.4 10.2-12.8	9.2 8.4-10.1	8.4 7.2-9.8	7.4 6.4-8.5	1.203
Do.....	Brown trout	4.7 4.1-5.3	4.4 3.9-4.9	4.4 3.9-4.9	4.4 3.9-4.9	1.230
Do.....	Brook trout	8.5 7.3-9.9	6.3 5.4-7.4	5.8 5.1-6.6	5.8 5.1-6.6	1.250
Do.....	Carp	--	22.0 21.8-22.2	15.0 14.9-15.1	13.3 13.2-13.4	1.182
Do.....	Fathead minnow	--	60.3 50.0-72.9	50.8 37.9-67.9	15.5 13.8-17.4	1.450
Do.....	Black bullhead	--	120.0 112.1-128.4	94.0 85.5-103.5	57.5 46.0-72.0	1.215
Do.....	Channel catfish	65.0 54.0-78.0	58.0 47.0-71.2	50.0 39.1-64.0	39.7 28.3-55.6	1.635
Do.....	Bluegill	--	7.9 6.5-9.3	7.1 6.0-8.4	7.1 6.0-8.4	1.473
Do.....	Yellow perch	19.5 14.7-26.0	12.3 10.5-14.4	10.0 8.1-12.3	10.0 8.9-11.2	1.505

trout were 6.3 for para-cresol, 7.9 for ortho-cresol, and 8.2 for meta-cresol. The data show that the isomers are only slightly more toxic to the salmonids at 96 hours than they are at 24 hours. Regarding all LC50's for the salmonids, a definite order of toxicity emerges for the three isomers: para-cresol is most toxic, followed by ortho-cresol, and finally meta-cresol.

Among the warm-water fish in bioassays with para-cresol, fathead minnows, black bullheads, and channel catfish proved to be most resistant. For these species as well as carp, the toxicity increased markedly in relation to time of exposure.

Observation during toxicity tests

During the first 10 minutes of the bioassays against brook trout at 6 to 20 ppm, the approximate incidences of surfacing were meta-cresol 20 percent, ortho-cresol 30 percent, and para-cresol 90 percent. The incidences for warm-water species exposed to para-cresol were carp 80 percent at 15 to 23 ppm, fathead minnows 30 percent at 30 to 150 ppm, bluegills 30 percent at 14 to 16 ppm, and yellow perch 50 percent at 12 to 18 ppm. Black bullheads and channel catfish did not surface at any concentration. Hemorrhages of the gills and at the base of the pectoral and/or pelvic fins were common in all bioassays.

Efficacy

Tests conducted at 10 ppm with the isomers of cresol in the lotic system confirmed the order of potency revealed in the static bioassays (fig. 2). Meta-cresol caused total incapacitation in 15 of 20 rainbow trout within 11.5 minutes, after which recovery to a higher level of activity occurred despite the continuing flow of chemical. Ortho-cresol induced total incapacitation in 17 of 20 fish within 15 minutes, with 3 fish remaining partially active. Para-cresol totally incapacitated 20 fish within 8.5 minutes.

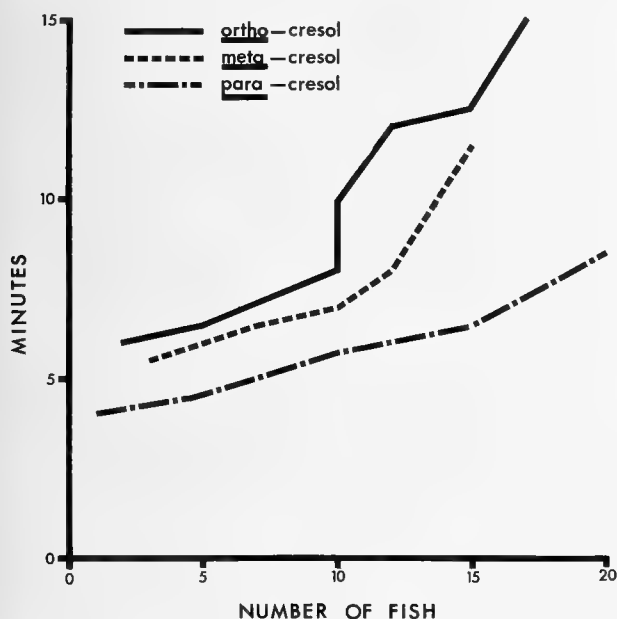


Figure 2.--Induction times and numbers of fish in total incapacitation from exposure to the isomers of cresol at 10 ppm.

Rainbow trout reacted violently upon contact with the isomers, as did all species tested in the static bioassays. Random lunging and thrashing occurred among the rainbows. Surfacing appeared during the throes preceding stupefaction but was not sustained. As the fish lost equilibrium, they became darker, the gular area reddened in some, and their bellies bloated as swimming activity diminished. Most fish settled to the bottom where blanched gills and several fin-base hemorrhages were noted. These responses were evoked quite uniformly by all three isomers.

The results of the flowing trials and static bioassays make it apparent that para-cresol is the prominent isomer for the incapacitation of rainbow, brown, and brook trouts. Thus it was decided to evaluate para-cresol in conjunction with certain compounds which are known to immobilize trout (Muench, 1958; Bové, 1962; Thienpoint and Niemegeers, 1965).

The concentrations of MS-222, quinaldine, and McNeil-JR-7464 which induce total incapacitation in 10 fish within specified times are presented in table 3. Flows and depths differed between these trials and those involving the isomers of cresol shown in figure 2. Therefore, the induction times of the two trials are not comparable. At least 7 minutes were required to attain the maximum concentration of a chemical in the test chamber, using malachite green as a tracer. Therefore, in some tests the fish never were exposed to the desired concentration, and in others they received the full dosage for only a portion of the time stated in the table. The calculated, ultimate concentrations achieved the desired effect in the times listed through buildup toward or to maximum level, as would be the case in a practical, stream application.

TABLE 3.--Concentration of four compounds necessary to induce total incapacitation in 9-inch rainbow trout within specified times

Time (minutes)	Number of fish	Concentration (ppm) of --			
		para-cresol	McNeil-JR-7464	Quinaldine	MS-222
4.5.....	10	--	3.0	37.5	110.0
5.....	10	--	2.5	35.0	95.0
6.....	10	--	2.3	25.0	80.0
7.....	10	30.0, 35.0, 40.0	2.2	20.0	65.0
8.....	10	22.4	2.0	17.3	56.3
9.....	10	35.0	1.8	15.0	--
10.....	10	14.0	1.7	--	--

Concentrations of para-cresol below 14.0 ppm required more than 10 minutes to induce total incapacitation; concentrations of 35 and 40 ppm did not improve on the induction time at 30 ppm (table 3). Repellency was strong at all concentrations. The incidence of surfacing was about 50 percent during partial incapacitation, but transitory with each fish. They settled to the bottom at the onset of total incapacitation.

The symptoms of stress, observed in the flowing trials of the isomers of cresol, were manifested again in these trials. Nine of 70 rainbow trout exposed to para-cresol developed hemorrhages at the base of pectoral and/or pelvic fins. Eight of 40 fish died as a result of the trials above 25 ppm. Recovery from incapacitation required 5.5 to 26.5 minutes, the longer times being associated with higher concentrations.

MS-222 was less efficacious than para-cresol and did not induce total incapacitation within 10 minutes at levels below 56.3 ppm (table 3). However, higher concentration were efficacious, although there was no improvement in reaction time at concentrations greater than 110 ppm. Neither repellency nor surfacing was observed, and no external signs of stress were visible. The time for recovery ranged between 4 and 10 minutes, and there were no deaths among 60 fish.

Quinaldine was effective in causing total incapacitation within 10 minutes at levels from 15.0 to 37.5 ppm (table 3). Concentrations below 15.0 ppm were not efficacious within 10 minutes; doses above 37.5 ppm did not improve significantly on reaction time. Moderate repellency was noted, and the rainbow trout darted about, shaking their heads and bodies. Only incidental surfacing occurred prior to total incapacitation. The only unusual manifestation in the stupefied fish was gyrating of the eyes. As recovery commenced, tremoring of the bodies was general. No deaths were recorded among 80 fish, and recovery required 5 to 14 minutes.

McNeil-JR-7464 was most effective between 1.7 and 3.0 ppm (table 3). There was no repellency and surfacing was only incidental before total incapacitation. Most of the fish assumed an inverted position while stupefied at the bottom. No signs of stress were evident at any stage of testing. The time for recovery ranged from 12 to 22 minutes, and no losses resulted among 50 fish.

DISCUSSION

Of the compounds tested, para-cresol fared poorly according to the criteria established for testing potential collecting compounds. It

caused violent repellency before rapid incapacitation and deaths of more than 10 percent. The active surfacing induced by para-cresol is not conducive to collecting fish as would a passive surfacing. Cresol-induced surfacing is a constantly shifting phenomenon which appears to be a vague effort to escape the irritating solution during the paroxysms which precede stupefaction.

There was considerable evidence of physiological disturbance from contact with the cresols at effective concentrations. Violent lunging and gulping was followed by bloating of the abdomen in most cases. Some fish reddened in the gular area, and others developed hemorrhages at the bases of the pectoral and/or pelvic fins. When the fish settled to the bottom, opercula and mouths were agape, and the gills were considerably blanched. They retained this appearance until death. These symptoms are similar to those described by Jones (1964) for phenol and cresols against perch.

As for the physiological damage which occurs, a Polish investigator has provided detailed accounts of phenol-induced changes in the blood and tissues of the bream Abramis brama (L.) (Waluga, 1966a, 1966b). Among his findings were quantitative changes in the blood, circulatory impairment, hemorrhages, cell necrosis leading to disintegration, and damage to the central nervous system; in summary, a general poisoning of the fish, damaging systems and organs essential to life.

The cresols are alkyl derivatives of phenol, and their systemic actions are so identical as not to warrant separation (Goodman and Gilman, 1965). It is apparent that the search for fish-collecting agents should be directed toward substances less harsh than the cresols.

MS-222 met the test criteria satisfactorily, although it did not induce surfacing. The relatively high concentrations required would make practical employment of this expensive drug unfeasible.

Quinaldine caused moderate irritation and repellency and no significant surfacing. This compound probably would be satisfactory as a collecting aid in small swiftly flowing streams,

where current action would wash the immobilized fish into a blocking seine. However, further data on the physiological effects of this compound are necessary.

McNeil-JR-7464 produced most of the desired effects on fish at very low concentrations. It should be effective for collecting in swift waters, and possibly in slower sections as well. The long recovery period and the inverted position of stupefied trout, exposing their light undersides, should enhance collection in nonturbid slow waters. This compound lacks only surfacing action to have excellent potential as a collecting tool. Its combination with a compound which induces only active surfacing might yield a valuable collecting tool.

CONCLUSIONS

1. Para-cresol is the most active of the three isomers of cresol in inducing total incapacitation of rainbow, brown, and brook trout.
2. The cresols are too harsh physiologically to warrant further development as fish-collecting agents.
3. MS-222 is not well suited as a fish-collecting tool; it does not induce surfacing, and a high concentration is necessary for total incapacitation.
4. Quinaldine may have some usefulness for collecting fish in small streams with rapid flow. Its physiological effects need elucidation.
5. McNeil-JR-7464 does not induce surfacing but is effective at low concentrations and appears to have desirable characteristics as a fish-collecting agent. It merits further investigation.

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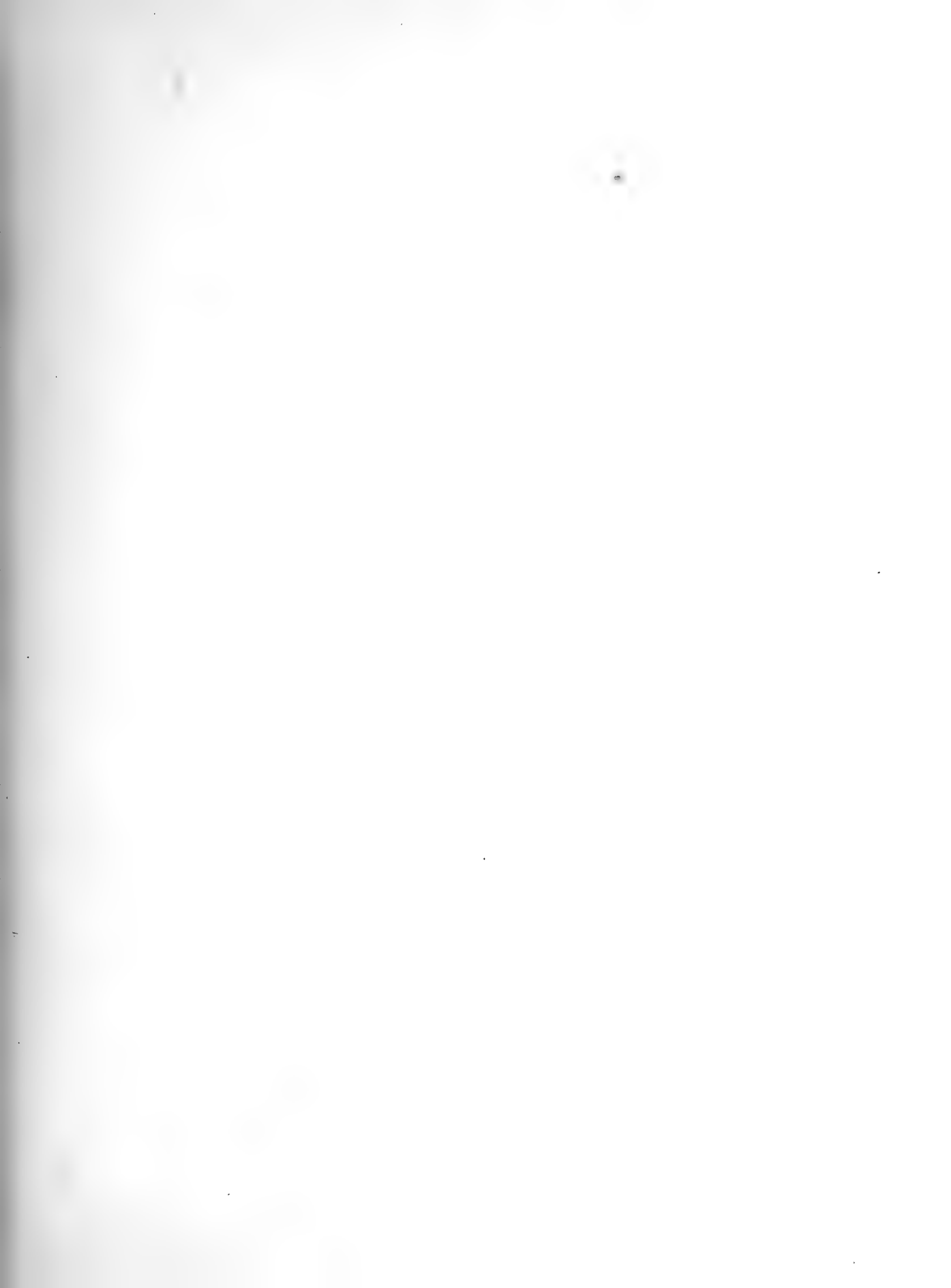
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Continued on inside back cover--

INVESTIGATIONS IN FISH CONTROL

**35. Toxicology of Thiodan
in Several Fish
and Aquatic Invertebrates**

By Richard A. Schoettger



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TOXICOLOGY OF THIODAN IN SEVERAL FISH AND AQUATIC INVERTEBRATES

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ABSTRACT.--This investigation provides toxicological data on Thiodan to meet published criteria for evaluating chemicals for fish control. Thiodan, a chlorinated hydrocarbon insecticide, was tested against rainbow trout and their fertilized eggs, western white suckers, *Daphnia magna*, and damselfly naiads. The median tolerance limits for trout and suckers ranged between 0.3 and 8.1 ppb and the fish were at least seven times more susceptible than the invertebrates. Toxicity was influenced by temperature, length of exposure, and alkaline pH. Exposures for up to 2 hours to 50 ppm of Thiodan were not toxic to fertilized trout eggs. The deposition and metabolism of Thiodan residues in western white suckers, northern creek chubs, and goldfish were traced with the aid of carbon-14 labeled Thiodan, and chemical analyses of Thiodan in tissues. Residues occurred in the skin and muscles of fish exposed to acute and to multiple subacute concentrations. The death of fish which were poisoned subacutely was correlated with size and the lipid content of muscle. The residues in the various tissues seemed to be associated with the method of treatment. A water-soluble metabolite of Thiodan in the bile of treated fish appeared to be a glucosiduronic acid conjugate of Thiodan alcohol. A possible metabolic pathway for Thiodan degradation is discussed. Thiodan appears to have little value as a selective piscicide against rough fishes such as carp or suckers, but under certain conditions it may be a good general fish toxicant.

Fishery biologists generally turn to the use of chemicals for manipulating fish populations when other methods of management such as seining, drawdown, draining, or biological controls are ineffective or too expensive. According to Stroud and Massman (1963) the principal chemicals used in fishery reclamations in the United States and Canada are the insecticides rotenone and toxaphene. They reported that another insecticide, Thiodan,^{1/} was applied experimentally to four small lakes in Canada. The trials indicated that it is highly toxic to fish, detoxifies more rapidly than toxaphene, and is less expensive than rotenone, but may be detrimental to plankton and bottom fauna.

Thiodan and toxaphene are classed chemically with the organochlorine or chlorinated camphene insecticides, which include aldrin, dieldrin, and endrin. It is generally recognized that this group of chemicals is relatively persistent in the environment. For example, toxaphene-treated waters may remain toxic to fish for 2 or 3 years (Tanner and Hayes, 1955; Hooper and Grzenda, 1955); residues of toxaphene may persist in fish tissues and in other parts of the ecosystem (Kallman, Cope, and

This paper is based on a dissertation submitted to the Faculty of the Zoology Department of Colorado State University in 1966, in partial fulfillment of the requirements for the degree of doctor of philosophy. Financial assistance from the National Institutes of Health is gratefully acknowledged.

^{1/} The term Thiodan (the trade name for endosulfan) is used in this paper because it is in common usage.

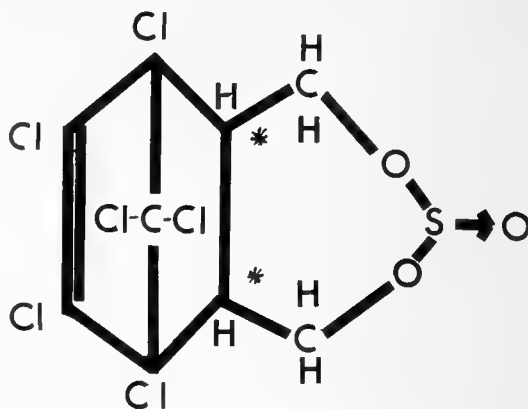
Navarre, 1962). The United States Department of the Interior has banned all use of this and other persistent chemicals in Department waters because of the effects on aquatic life. Some question remains whether Thiodan should be considered for fishery uses because of its chemical similarity to toxaphene. Recently Meyer (1965) reported on the organophosphorus insecticide Guthion as a potential fish poison less persistent than organochlorine insecticides. The rapid degradation of antimycin A (a piscicide registered under the tradename of Fintrol) shows promise for fish management (Walker, Lennon, and Berger, 1964). Perhaps a variety of fish control agents with well-defined chemical and toxicological properties will enable biologists to select suitable compounds for specific management problems.

Thiodan was patented in the United States as an insecticide by Frensch et al. (1957) and later in Germany as a fish toxicant (Frensch et al., 1959). Cuerrier (1960) made field observations on the efficacy of Thiodan in fish control. Adlung (1957) and Lüdemann and Neumann (1962) measured its relative toxicity to several aquatic vertebrates and invertebrates. However, these data are insufficient to meet the criteria, as established by Lennon and Walker (1964), for gaging the potential of chemicals for fish control. The objective of my investigation was to provide additional information on Thiodan by determining its toxicity to other fish and aquatic invertebrates, the effect of pH and water quality on toxicity, and its uptake and metabolism in fish.

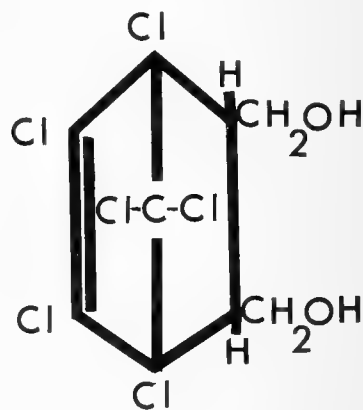
MATERIALS

Thiodan is the registered tradename of a chlorinated fused-ring heterocyclic compound developed by Farbwerke Hoechst A. G., Frankfurt, Germany, as an insecticide (F.M.C. Corporation, 1964). The Food Machinery and Chemical Corporation (F.M.C.), Niagara Chemical Division, Middleport, N.Y., is licensed to manufacture, use, and sell Thiodan in the United States. The American common name of Thiodan is endosulfan (6,7,8,9,10,10-hexachloro 1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide).

The empirical formula is $C_9H_6Cl_6O_3S$ and its structure is as follows (F.M.C. Corporation, 1958a and 1964):



According to the manufacturer, the technical Thiodan used in this study is 96.4 per cent pure. It contains A and B isomers which melt in the ranges of 108° to 109° C., and 206° to 208° C., respectively (Lindquist and Dahm, 1957). The acid hydrolysis of either isomer yields only one isomer of Thiodan alcohol which has the structure:



Thiodan is insoluble in water and stable in sunlight, but hydrolyzes to sulfite and Thiodan alcohol in the presence of moisture, bases or acids. Other physical and chemical properties are reported by the maker (F.M.C. Corporation, 1964).

The F.M.C. Corporation supplied approximately 17 milligrams of carbon-14

labeled Thiodan for tracer investigations. It has an activity of 5.91 microcuries (μc) per mg. Carbon-14 constitutes 6.8 percent of the molecule and 25 percent of the total carbon. The locations of carbon-14 in the Thiodan molecule are shown by asterisks in the structural formula. The synthesis of radioactive Thiodan was outlined by Forman et al. (1960).

METHODS

Toxicity

Thiodan was bioassayed against small rainbow trout and western white suckers and against *Daphnia magna*, damselfly naiads and fertilized eggs of rainbow trout (table 1). Most of the experiments were conducted in the Zoology Laboratory, Colorado State University, Fort Collins, Colo., but the egg trials were carried out at the Fish Control Laboratory, La Crosse, Wis. The fish were maintained in aerated tap water at 10° C. If deaths in a particular lot of fish exceeded 5 to 10 percent, it was discarded. Both species were held 10 days before use and were fed Colorado's standard pelleted diet. They were fasted for 48 hours and acclimated to experimental temperatures for 24 hours before the trials. The naiads were acclimated to laboratory conditions for several days before use.

The bioassays were conducted according to procedures described by the American Public Health Association (1960), with some modifications. Trials with fish were in glass aquariums containing 20 liters of tap water; those with damselfly naiads were in 6 liters. Experiments with *D. magna* were in plastic vessels each holding 1 liter of reconstituted water. In the latter, tap water was deionized and formulated to contain 45 to 50 ppm of bicarbonate alkalinity with sodium bicarbonate, and 45 to 50 ppm of calcium chloride. Chemical analyses of the tap water showed it to contain the following: Oxygen 11.8 ppm (7° C.); total hardness 45.0 ppm as calcium carbonate; total alkalinity as bicarbonate 38.0 ppm as calcium carbonate; calcium 12.0 ppm; magnesium 2.3 ppm; ferric and aluminum oxide 1.6 ppm; sulfate 1.3 ppm; chloride 6.9 ppm; chlorine 0.0; and pH 7.4.

Desired concentrations of Thiodan were prepared by adding aliquots of 1-part-per-thousand stock solutions of technical Thiodan in ethanol to the water. The concentrations were based on active ingredient. Ethanol was added to controls in an amount equal to the largest aliquot of stock used in the test series. A minimum of aeration was applied to all of the solutions tested against fish to maintain an oxygen concentration of not less than 5 ppm. Ten fish and 10 or 20 invertebrates were used to measure toxicity, and the trials were replicated 1 to 4 times. The loadings of fish in test vessels were approximately 0.5 to 1.0 gram per liter.

The bioassays with each species were carried out at two temperatures: Trout, 1.5° and 10° C.; suckers, 10° and 19°; *D. magna*, 10° and 19°; and naiads, 8° and 19°. Temperature control was achieved by manipulating room temperature. At 10° and below, temperature varied $\pm 1^\circ$ C., and at 19° the fluctuation was $\pm 2\text{--}3^\circ$.

Observations on the toxicity of Thiodan were made 24, 48, 72, 96, and 120 hours after the experimental animals were first exposed. Individuals showing no respiratory movement and no response to a tactile stimulus were recorded as dead and were removed.

Between experiments, test containers were cleansed with detergent, filled with 5- to 10-percent potassium hydroxide, allowed to stand several hours, washed again, and then rinsed with ethanol and dried. This procedure removed or destroyed Thiodan which adhered to the glass as indicated by periodic bioassays of cleansed aquariums.

The median tolerance limits (TLm, the concentration tolerated by 50 percent of the test animals) of trout, suckers, naiads, and *D. magna* to Thiodan were determined for each temperature and observation period. A polynomial quadratic equation was derived from the data for the 24- and 48-hour bioassays as described by Goulden (1956). TLm was interpolated from the regression line. The 95-percent confidence limits around the TLm's were computed from the standard errors for the populations.

Table 1:--Species, sizes, and sources of experimental animals.

Species	Size range		Source
	Length (inches)	Weight (grams)	
Rainbow trout, <u>Salmo gairdneri</u>	1.6-2.2	1.0-1.8	SFH ^{1/} , Bellvue, Colo.
Western white suckers, <u>Catostomus commersoni</u>	1.8-2.6	0.9-2.5	Cache la Poudre River, Fort Collins, Colo.
Do.	5.3-8.2	40.1-70.0	
Northern creek chubs, <u>Semotilus atromaculatus</u>	6.7 ^{2/}	48 ^{2/}	
Goldfish, <u>Carassius auratus</u>	6.7-10.9	96-337	NFH, Genoa, Wis.
Rainbow trout (fertilized eggs)	---	---	NFH, Manchester, Ia.
Water fleas, <u>Daphnia magna</u>	---	---	Laboratory cultures at Colorado State Univ., Fort Collins, Colo.
Damselfly naiads, <u>Ischura</u> sp.	0.25-0.50	---	From beneath the ice of a pond near Fort Collins, Colo.

^{1/} State fish hatchery.^{2/} Mean.^{3/} National fish hatchery.Solutions for testing water quality effects on toxicity

The TLM's for 72, 96, and 120 hours were interpolated from averages of the replicate bioassays by methods of the American Public Health Association (1960).

Eggs of rainbow trout were fertilized at the National Fish Hatchery, Manchester, Iowa, and transported to the laboratory. Twenty-five hours later they were divided into groups of 100 each and dipped into solutions containing 10, 100, 1,000, 10,000, or 50,000 ppb of Thiodan for 30 or 120 minutes. Test solutions were prepared with technical Thiodan dissolved in acetone, and control solutions with a corresponding concentration of acetone alone. After dipping, the eggs were placed in a Heath Incubator at 12° C. for hatching. At 24-hour intervals the eggs were observed for mortality, and 2 or 3 specimens were collected randomly for microscopic examination. The samples were prepared for the examination, and stages of development were identified as described by Knight (1963).

Salt solutions were made to a hardness of 500 ppm, as calcium carbonate, by adding calcium chloride, calcium sulfate, magnesium chloride, or magnesium sulfate to 3 liters of tap water in 1-gallon glass jars. Thiodan dissolved in ethanol was added to make a concentration of 20 ppb. Controls were salt solutions without the toxicant, solutions with Thiodan, but no salts, and vessels with only tap water.

Effects of pH on toxicity were measured in solutions of pH 6.4, 7.4, 8.4, and 9.4. The pH was adjusted by adding potassium hydroxide and/or acetic acid to 3 liters of tap water in 1-gallon glass jars. A concentration of 20 ppb of Thiodan was added to each jar. Control solutions had pH values appropriate for a particular pH test, but were without Thiodan.

Following preparation of experimental media, the glass jars were sealed and the contents aged at 19° C. + 2° to determine degradation time. Salt solutions were aged up to 436 hours, and solutions of different pH for up to 96 hours. Then they were aerated and bioassayed for 24 hours with 10 suckers with an average length of 2 inches and weight of 1.3 grams.

The comparative differences in toxicities of the solutions were analyzed statistically by the exact test for 2 x 2 tables (Goulden, 1956).

Deposition and metabolism of Thiodan

Radioactive Thiodan--Western white suckers were bioassayed in 1-gallon glass jars containing 1.5 or 3.0 liters of tap water at 19° C. One sucker was placed in each jar per 1.5 liters of solution. The fish had an average length of 5.3 inches and weighed 40.1 grams. Radioactive Thiodan was dissolved in ethanol, and a stock was prepared as in the toxicity experiments. Portions of this stock were added to the jars to give concentrations of 20 and 80 ppb of labeled compound. At the time of these experiments, the exact purity of the labeled Thiodan was unknown. Forman et al. (1960) later reported that it contained 85- to 93-percent Thiodan, but the impurities were not radioactive. Thus, the amount of pure Thiodan in each jar was slightly less than 20 or 80 ppb.

The fish exposed to a concentration of 80 ppb were removed for analysis at the time of death. Those treated with 20 ppb were killed with chloroform at intervals between 1 and 2 hours of exposure. Samples of gill filament, heart, liver, kidney, skin, muscle, gut (with and without feces), brain, and blood were collected. Blood was obtained by cardiac puncture immediately after death. The samples, excluding blood, were rinsed briefly in distilled water, placed in tared vials, and dried at 90° to 100° C. to constant weight. They were digested in 3.0 ml of 2-percent potassium hydroxide. A 0.5-ml aliquot of each digest was placed on a tared stainless steel planchet of approximately 20 cm and diluted with deionized water. A homogeneous distribution of the material on the planchet was promoted by stirring in a small quantity of laboratory detergent which reduced surface tension. This also reduced contraction of the samples on the planchets while they dried to constant weight. The samples were then ready for measurement of radioactivity.

Determinations of radioactivity were made with an internal proportional counter and

the counts corrected for background and counter efficiency. Characteristics of the counter were reported by Nader, Hagee, and Setter (1954). The 0.95 counting error was computed for each sample according to the nomograms of Jarret (1946). Concentrations of Thiodan greater than the counting error were considered significantly different from zero. Untreated control fish were also counted, but none contained statistically significant radioactivity.

Radioactive Thiodan was used to determine whether and how it may be metabolized in fish. Large northern creek chubs and western white suckers were exposed to 40 ppb of labeled Thiodan at 21° C. using methods and materials described in the radiation studies. The fish were killed with chloroform after 3 or 5 hours of exposure.

Samples of chub liver, kidney, blood, and brain were dried to constant weight at 90° C., macerated, and extracted in absolute ethanol. The ethanol was evaporated almost to dryness at room temperature, and the residues were washed with alternate rinses of benzene and deionized water into 125-ml separatory funnels. The mixtures were agitated and allowed to separate for 1 to 12 hours, depending on persistence of emulsions. The emulsions persisted with all samples, except brain. Aliquots of benzene or water fractions were placed on planchets and dried for measurements of radioactivity. Small amounts of persistent emulsions were included with the water fraction. The counts were corrected for background, geometry and backscatter, but not absorption since the amount of lipid per planchet was usually less than 0.2 mg.

The tissues which had been extracted with alcohol were prepared for analyses of radioactivity as described earlier for potassium hydroxide digests of sucker tissue.

The gallbladder bile of chubs and suckers was dried to constant weight at 65° C. and rehydrated in 30-percent ethanol in water. Aliquots of the rehydrated bile were washed with benzene and water into separatory funnels, separated, and analyzed for radioactivity like the alcoholic extracts of chub tissues. Additional aliquots

were diluted to 10 ml with deionized water. Approximately 1 ml of 0.1M sodium acetate and 3.0 ml of 0.2M acetic acid were used to buffer the bile to pH 4.5. The buffered mixtures were incubated for 24 hours at 37° C. with 0.5 ml (2,500 Fishman units) of beta-glucuronidase,^{2/} a procedure similar to that described by Talalay (1963). The samples were raised to pH 7.5, then extracted with benzene, and the levels of benzene- and water-soluble radioactive substances were determined.

After approximately a 6-month delay in the research, a check was made of the molecular integrity of the stock supply of radioactive Thiodan. The results of paper chromatographic analyses, methods according to Mills (1959), indicated that the labeled chemical had degraded to what appeared to be Thiodan alcohol. Experiments with radioactive Thiodan were discontinued.

Nonradioactive Thiodan-- Further investigations on the deposition and metabolism of Thiodan in fish were conducted at the Fish Control Laboratory, La Crosse, Wis. Goldfish were maintained in flowing well water at 12° C. and fasted for 24 hours before the tests. An individual was placed in each of 10 glass jars containing 15 liters of reconstituted water at 12° C. The water was reconstituted by adding various salts to deionized water, methods according to Lennon and Walker (1964). Stock solutions of technical Thiodan were prepared in acetone and an aliquot containing 0.105 mg. of toxicant was added to each test solution to give a concentration of 7.0 ppb. Control fish were exposed to similar quantities of acetone. The living test and control fish were transferred daily into new test solutions. The solutions were aerated periodically to insure adequate oxygen.

The goldfish experiment was terminated after 21 days. All of the fish, except two, died during the tests and were removed for tissue analyses. The two survivors were killed by

overanesthetization in MS-222. The gill filaments, gonads, livers, spleens, hearts, kidneys, guts (including feces), skin, muscle, brains, peritoneal fat (from two specimens), scales, and fins were dissected from the dead fish. Heparinized samples of blood were collected by cardiac puncture, and the gallbladder was removed from the liver.

According to preliminary tests using analytical methods which are outlined later, I found it necessary to combine tissues, except muscle, from several fish. Samples of approximately 10 to 50 grams were needed for the analyses. The gills from the first five fish which died were combined into one sample, those from the last three and the two survivors into another. Samples of the skin, liver, gut and content, gonad, and two composite samples, one of the heart, kidney, spleen, and blood and another of the scales and fins were prepared like the gill. All of the brains were pooled into one sample. Peritoneal fat was dissected from two fish and pooled. The various samples were weighed and combined immediately after dissection. They were weighed again, after drying to constant weight at 70° C., to determine water content. The gallbladder bile was measured volumetrically and set aside for other analyses. The dried tissues were extracted with petroleum ether for 5 to 6 hours in a Soxhlet apparatus. The lipid content was determined by allowing the petroleum ether to evaporate and weighing the residue. They were then redissolved in the same solvent.

The extracts were analyzed for Thiodan by the colorimetric procedure described by Maitlen et al. (1963). He observed that captan, chlordan, heptachlor, and ovex caused some interference in the method, but 45 pesticides and several chlorinated solvents did not. The method is applicable for 5 to 50 μ g of Thiodan and also measures Thiodan alcohol. The analyses were made on a Beckman Model DB spectrophotometer.

Before Thiodan can be analyzed colorimetrically, it must be separated (cleaned up) from lipids in ether extracts. Cleanup was

^{2/} Ketodase. Warner-Chilcott, Morris Plains, N. J.

accomplished by acetonitrile-petroleum ether partition, and by chromatography on a 60/100 mesh, activated Florisil-carbon column described by Murphy and Barthel (1960).

Efficiency of the cleanup technique was determined by running Thiodan standards, or known amounts of Thiodan added to fish fat through the procedure. The columns were eluted with 8-percent diethylether in petroleum ether. The overall recovery averaged 56.3 percent and varied approximately 3 percent. The average recovery was used to correct measurements of Thiodan in fish tissues. Maitlen et al. (1963) obtained recoveries of approximately 77 percent, and Moats (1963) indicated that only one isomer of Thiodan is eluted from Florisil. Thiodan alcohol was recovered completely after acetonitrile partition, but, as was also reported by Moats (1963), none could be eluted from the Florisil column.

In spite of cleanup, low levels of lipids persisted in Thiodan-fish fat standards and in the tissue samples, particularly those from muscle. The lipids did not appear to significantly alter absorbance or wavelength. Analyses of standards containing even greater lipid levels and known amounts of Thiodan supported the observations.

The bile samples were composed of bile from two fish which died on the same day, or successive days. For example, the bile of fish which died on the fourth and fifth days of treatment were combined into one sample, that from individuals dying on the seventh and eighth days formed another, and so forth. The samples were extracted with petroleum ether in a separatory funnel before and after incubation with beta-glucuronidase, as in the radiation experiments. The extracts were analyzed colorimetrically for Thiodan.

RESULTS AND DISCUSSION

Toxicity

Fish--The toxicity of Thiodan to rainbow trout and western white suckers is influenced by

temperature and exposure. The 24- to 120-hour TLm's for trout at 1.5° and 10° C. range from 5.9 to 0.7 ppb and 2.1 to 0.3 ppb respectively (table 2). The TLm ranges for suckers at 10° and 19° are 8.1 to 2.5 ppb and 6.6 to 2.8 ppb (table 2). The results show that in general the toxicity of Thiodan to these species of fish increases with warmer temperatures and longer exposures.

The relation between toxicity and temperature and exposure is shown graphically in figure 1. In general, toxicity increases significantly up to approximately 72 or 96 hours and suggests that the toxicity is associated with accumulations of Thiodan in excess of the amounts that may be metabolized or stored by the fish. Relatively small changes in toxicity occurred with additional exposure (96 and 120 hours), with the exception of suckers at 10° C. The plotted line for suckers at 10° is linear whereas that for trout is curvilinear. The latter species may be able to metabolize Thiodan more efficiently at 10° than the former, though not in equivalent amounts since suckers were more resistant.

The nonlinearity of several of the curves in figure 1 may also be related to the amount of toxicant available for uptake by fish in static tests such as these. Holden (1962) showed, for example, that within 10 hours 80 to 90 percent of the radioactive DDT in static tests was taken up by the fish, detritus, or on the sides of the containers. It is possible that constant-flow tests, or tests where the toxicant is periodically renewed would reveal greater toxicity over longer exposures.

Extrapolations of the 24- and 48-hour regressions used to determine TLm's for trout and suckers indicate that, with one exception, there is less effect of temperature and exposure on the concentrations necessary for 100-percent mortality than on the TLm's. The 100-percent lethal levels for trout are approximately 3 to 5 ppb and 8 to 10 ppb for suckers. The 24-hour concentration of 9.5 ppb for trout at 1.5° C. is exceptional. Adlung (1957) observed that temperature had little effect on the "absolute lethal dosage" of Thiodan to goldfish.

Table 2:--Toxicity of Thiodan to two species of fish and aquatic invertebrates at two temperatures [Median tolerance limits in parts per billion; 95-percent confidence intervals in parentheses].

Species and temperature	At 24 hours	At 48 hours	At 72 hours	At 96 hours	At 120 hours
Rainbow trout:					
At 1.5° C.	5.9 (5.2-6.6)	2.1 (1.4-2.8)	1.4 ---	0.8 ---	0.7 ---
At 10° C.	2.1 (1.5-2.7)	1.1 (0.7-1.5)	0.4 ---	0.3 ---	0.3 ---
Western white sucker:					
At 10° C.	8.1 (7.4-8.8)	6.4 (5.6-7.2)	4.9 ---	3.5 ---	2.5 ---
At 19° C.	6.6 (5.9-7.3)	4.3 (3.3-5.3)	3.1 ---	3.0 ---	2.8 ---
<u>Daphnia magna</u> :					
At 10° C.	178.0 (162-194)	132.0 (113-151)	87.5 ----	52.9 ----	47.5 ----
At 19° C.	68.0 (54-82)	62.0 (49-75)	60.5 ----	56.0 ----	53.5 ----
Damselfly naiads:					
At 8° C.	235.0 (165-305)	120.0 (65-180)	84.5 ----	71.8 ----	62.0 ----
At 19° C.	275.0 (240-310)	175.0 (135-215)	150.0 -----	107.0 -----	75.0 ----

The laboratory and field trials with Thiodan reported by other investigators show that it is highly toxic to a variety of species. Adlung (1957) killed goldfish with 10 ppb in 20 hours at 19° to 22° C. Lüdemann and Neumann (1960a) determined a 48-hour LC50 of 11 ppb for carp (Cyprinus carpio) at 17° to 19°. Relative to other organochlorine insecticides, they found that endrin was more toxic by a factor of two and toxaphene less toxic by a factor of five. Also, they later reported that 10 and 5 ppb killed all rainbow trout and northern pike (Esox lucius) respectively within 48 hours at 19° to 21° (Lüdemann and Neumann, 1961a).

F. M. C. Corporation (1958b) applied 46 ppb of Thiodan to a 27-acre pond and killed all minnows, perch, sunfish, bullheads, and suckers within 7 days. Guerrier (1960) was able to eliminate bluntnose minnows, golden shiners, common suckers, bullheads, perch, smallmouth bass, and sunfish from a lake with a concentration of 15 ppb. In both field trials, there was mortality of frogs and aquatic insects. Plankton declined, but later recovered.

The symptoms of Thiodan poisoning in trout and suckers are similar to those described by other investigators for fish exposed to organochlorine insecticides (Adlung, 1957; Henderson et al., 1959; and Lüdemann and Neumann, 1960a and 1961a). The fish seem overly excitable at first and swim rapidly about the aquariums. Later they surface, lose equilibrium, and move with spasmodic jerks. In time, the majority sink to the bottom, and opercular movements become erratic. Many of the trout become darker, and the suckers appear mottled. Death follows after a variable period.

Some of the trout which survived the 120-hour bioassays at 1.5° C. were placed in fresh aerated tap water. With tactile or vibrational stimuli, many individuals showed symptoms of Thiodan poisoning. Few lived longer than 5 to 7 days after the transfer. Lüdemann and Neumann (1960a) found that the effects of Thiodan on carp, with one exception, were irreversible. One carp exposed to 14 ppb

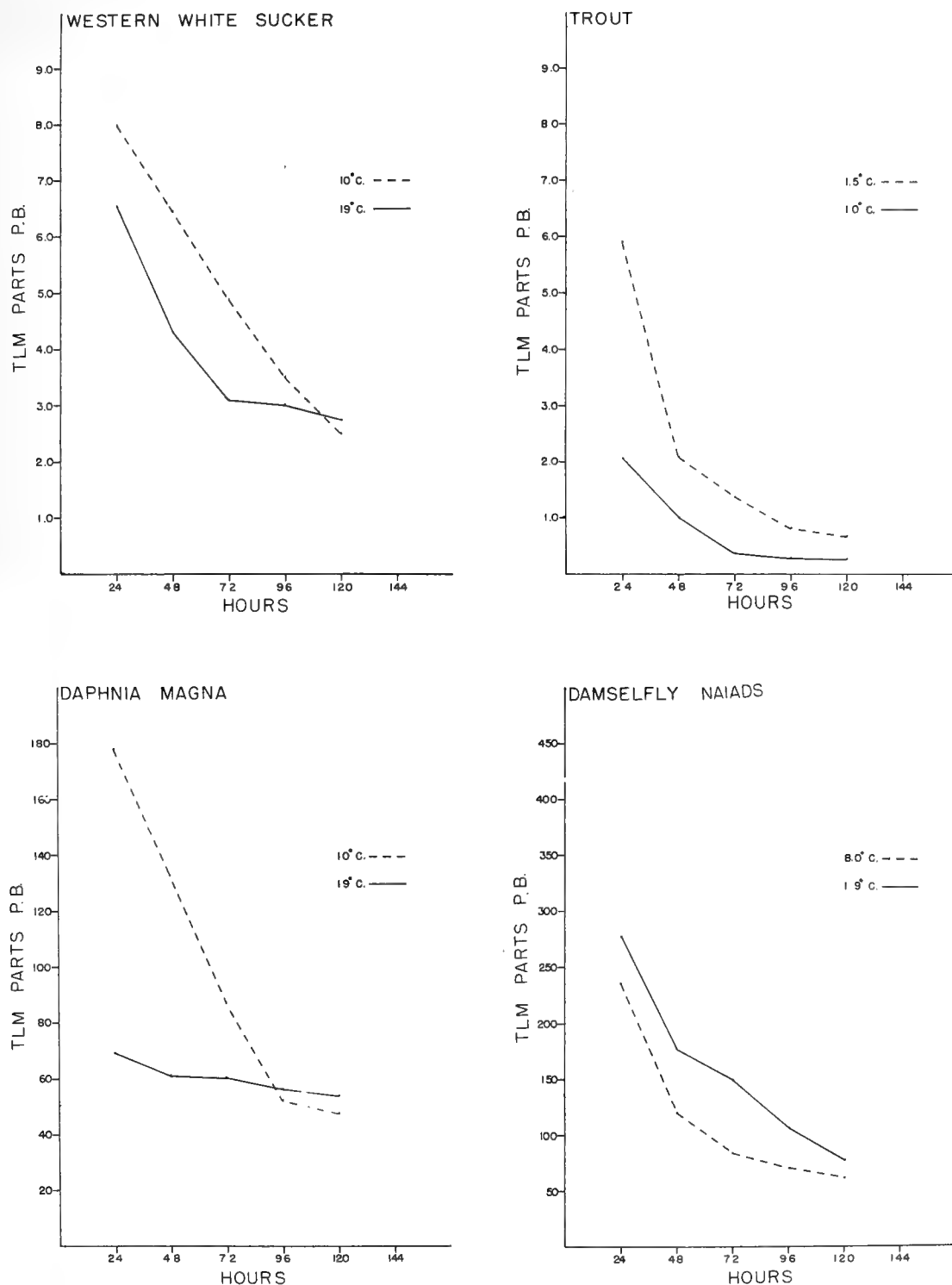


Figure 1:--Effects of time and temperature on the TLM's of rainbow trout, western white sucker, Daphnia magna, and damselfly naiads to Thiodan.

lay on its side after 40 hours, but recovered completely in fresh water.

Thiodan is one of the more toxic chlorinated insecticides to fish, but it is one of the least toxic to mammals. Linquist and Dahm (1957) reported an acute oral LD50 of Thiodan in corn oil to rats of 40 to 50 mg per kg body weight. The manufacturer obtained a value of 110 mg per kg when the insecticide was administered orally in cottonseed oil, and an LD50 of 359 mg per kg when it was applied to the skin of rabbits (F.M.C. Corporation, 1964). Negherbon (1959) cited oral toxicity values in mg per kg for similar chlorinated insecticides dissolved in peanut oil: endrin 16.8; dieldrin 38.3; toxaphene 40.0; and aldrin 45.9.

Loeb (1963) determined the oral toxicity of Thiodan to carp. Technical Thiodan was not toxic in 120 hours at doses of 119 to 234 mg per kg, but doses of 49 and 195 mg per kg of 24-percent miscible Thiodan were lethal within 118 and 22 hours respectively. A dust form of the insecticide was twice as toxic as the miscible formulation. The lethal oral levels for carp are similar to the toxic doses for rats. Although the doses for carp cannot be compared directly with lethal concentrations in water, they suggest that Thiodan is more easily absorbed across gill and oral membranes than by the intestine.

According to my results, and those of others, Thiodan appears to have little value as a selective piscicide against suckers or carp. Concentrations which are lethal to so-called rough or problem species are also toxic to game fish such as trout and northern pike. Fishery biologists recognize, however, that in many instances where fish control is indicated, the proportion of game fish may be insignificant. Here, a nonselective poison such as Thiodan could be used provided it was not overly detrimental to other aquatic organisms and decomposed rapidly into a harmless product.

Fertilized fish eggs--The exposure of rainbow trout eggs to various concentrations of Thiodan for 30 or 120 minutes appears to have

no adverse effect on their hatching success (table 3). Development in the eggs had progressed to approximately the 32- or 64-cell stage at the time of treatment. The eggs hatched 29 days after fertilization, and mortalities in the control groups were similar to those observed in groups exposed to concentrations of up to 50,000 ppb. I was unable to test higher concentrations because Thiodan could not be maintained in the test solutions. It formed a noticeable precipitate in the water and adhered to the sides of the containers.

Neither gross nor microscopical observations of treated and control eggs revealed any abnormal development that was attributed to exposure to Thiodan. Large numbers of young fish in the test and control groups died 46 days after hatching. Fish from the same lot of eggs, but used in other experiments at the Fish Control Laboratory, also died. Some factor other than Thiodan was believed responsible for the mortality, although a causative agent could not be established.

Rainbow trout eggs are extremely resistant to Thiodan in comparison with 2-inch fish of the same species. This resistance may be related to selective permeability of the egg chorion or to underdevelopment of Thiodan-sensitive structures at the time of treatment, or both. Antimycin A, a registered fish toxicant which inhibits oxidative-phosphorylation (Walker, et al., 1964), was tested against eggs from the same lot as those in the Thiodan trials. It killed the eggs at a concentration of 10 ppb, which suggests selective permeability of the egg chorion, or at least a mode of action different from that of Thiodan. Exploratory tests with Thiodan and antimycin against the eggs of northern pike support this finding.

Berger^{3/} also tested Thiodan against eggs of rainbow trout, but exposed them continuously to the toxicant until they hatched.

^{3/} Personal communication from Bernard L. Berger, Chemist, Bureau of Sport Fisheries and Wildlife, Fish Control Laboratory, La Crosse, Wis., 1965.

Table 3:--Hatching success of rainbow trout eggs after 30- or 120-minute exposures to various concentrations of Thiodan.

Concentration and exposure	Number of eggs	Number unfertilized	Number examined microscopically	Number remaining before hatching	Percent hatching
Control:					
30 minutes	100	1	35	64	76.6
120 minutes	100	7	28	65	70.8
10 ppb:					
30 minutes	100	5	31	64	84.4
120 minutes	100	5	29	66	69.7
100 ppb:					
30 minutes	100	7	31	62	51.6
120 minutes	100	6	29	65	86.2
1,000 ppb:					
30 minutes	100	7	31	62	79.0
120 minutes	100	2	28	70	68.2
10,000 ppb:					
30 minutes	100	6	31	63	76.2
120 minutes	100	0	28	72	94.4
50,000 ppb: ^{1/}					
30 minutes	100	3	32	65	73.8
120 minutes	100	3	28	69	85.5

^{1/} Thiodan formed a white precipitate in these solutions.

The hatched fish were held in the same solutions. He found that levels up to 50,000 ppb were not lethal over the 25-day incubation period, but that the fish became more susceptible after hatching and as they grew and absorbed the yolk sac. Concentrations of 750, 500, and 250 ppb caused 100-percent mortality of trout fry after 7, 12, and 20 days respectively. None of the treated fish survived after 30 days in 100 ppb of Thiodan, although about 30 percent of the controls also died at this time. Iyatomi et al. (1958) observed similar toxicity of endrin to newly hatched carp and snakehead fish (Channa argus).

Daphnia magna--Lüdemann and Neumann (1960b, 1960c, 1961b, and 1962) and Cuerrier (1960) indicate that most aquatic invertebrates, including Daphnia, are less sensitive than fish to Thiodan. My bioassays support this finding (table 2). The 24- to 120-hour TLM's at 19° C. range from 68.0 to 53.5 ppb, and at 10° the values are 178 to 47.5 ppb. The lowest value at 10° indicates that D. magna are at least six

times as resistant to Thiodan as suckers or trout. Thiodan used in fish control at concentrations of approximately 50 ppb, as it was in one experimental field trial, could have an adverse effect on Daphnia populations.

Lüdemann and Neumann (1960b) suggest that D. magna are even more resistant to Thiodan than was indicated by my bioassays. They found that 100 ppb caused only partial mortality, and 1,000 ppb killed all of the animals in 24 hours at 18° to 21° C. Extrapolation of the regression for the 24-hour TLM at 19° yields a 100-percent lethal concentration of about 90 to 100 ppb. Since they did not measure the toxicity of concentrations between 100 and 1,000 ppb, levels considerably below the latter value also may be 100-percent lethal.

Thiodan does not appear to have a significant chronic effect on D. magna at 19° C. The TLM values change little with longer exposures (fig. 1). The chemical degradation of Thiodan is a possible explanation. The addition of

sodium bicarbonate to the media containing Daphnia increased pH to about 7.9. In later tests pH 8.4 promoted decomposition of Thiodan, and conceivably pH 7.9 had a similar effect.

At 10° C. Thiodan is initially 2.5 times less toxic to D. magna than at 19° (fig. 1). With longer exposures the toxicity of the compound increases and after 96 hours gives TLM values similar to those at the higher temperatures. The chronic effect of Thiodan at 10° suggests that degradation at alkaline pH is temperature dependent.

Damselfly naiads--Damselfly naiads appear to be the most resistant to Thiodan of the four species of animals tested, except for trout eggs. However, in these trials the chemical was more toxic to naiads in cold water than in warm water. The TLM's at 8° C. ranged from 235 to 62 ppb, whereas at 19° the values were 275 to 75 ppb (table 1). In tests with other species, the compound is usually less toxic at colder temperatures. The greater toxicity of Thiodan to naiads at 8° was unexpected, but a second series of tests supported the earlier results. Since the bioassays extended over a period of 4 to 5 weeks, older naiads were used in the 8° trials. These specimens may have been physiologically more susceptible to Thiodan than younger individuals. McDonald and Jacobson (1958) demonstrated differences in the susceptibilities of army cutworms of various ages to endrin. They found that older larval instars were more resistant. The older damselfly naiads in my trials may have been less resistant. Thiodan appears to have a chronic effect on the insects with time, but levels off at about 60 to 70 ppb after 120 hours of exposure at both temperatures (fig. 1).

According to the results of Stringer and McMynn (1956), and Schoettger and Olive (1961) toxaphene appears relatively more toxic than Thiodan to damselfly naiads. They found that levels of 10 to 30 ppb of the former compound were lethal. However, insects such as Chironomus and Corethra (Chaoborus) are more sensitive to Thiodan than to toxaphene (Lüdemann and Neumann, 1962).

Extrapolations of the 24 - and 48-hour regressions indicate that approximately 425 ppb of Thiodan are required to kill 100 percent of the damselfly naiads. This concentration is relatively independent of temperature and exposure.

Effects of water quality on toxicity

Calcium and magnesium salts--Salts of calcium and magnesium in concentrations of 500 ppm, as calcium carbonate hardness, do not alter the toxicity of 20 ppb of Thiodan to suckers (table 4). The mortalities of fish in solutions containing salts and Thiodan, and in those without salts are similar and appear unrelated to aging of the solutions. Statistically, the probability of observing this set of data under the null hypothesis is 0.81. Solutions containing only the salts are not toxic.

pH--Trials with D. magna suggested that alkaline pH may influence the toxicity of Thiodan. Solutions containing 20 ppb of Thiodan were adjusted to pH 6.4 or 8.4 and bioassayed with suckers at 19° C. The results in table 5 indicate that pH has no effect on toxicity. Since the solutions were open to the air and aerated, pH was measured periodically. It declined about 2 to 4 tenths of a pH unit in both acidic and basic solutions during the first few hours of the test. After 24 hours the pH of acidic solutions was approximately 6.2 and that of the basic solutions was 7.4. Although pH control was unsatisfactory, the insecticide did not degrade rapidly in the early segment of the test.

The later determinations of effects of pH on the toxicity of Thiodan were performed by adjusting pH of the solutions and then sealing and storing them for various periods before bioassay. The change in pH was less than 0.75 of a unit over periods of 240 hours using this technique. The chemical degradation of Thiodan with time rather than the immediate effect of pH on toxicity was measured.

The storage of solutions containing 20 ppb of Thiodan at pH 6.4 for periods up to 240 hours has no effect on their toxicity to suckers (table 6). A statistical evaluation of

Table 4:--Effects of calcium and magnesium salt solutions, and solution age, on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay			Number of fish surviving in--								
			Tap water containing 500 ppm of				20 ppb of Thiodan	20 ppb of Thiodan and 500 ppm of			
			CaCl ₂	MgCl ₂	CaSO ₄	MgSO ₄		CaCl ₂	MgCl ₂	CaSO ₄	MgSO ₄
Number of fish per jar	Tap water control										
48 hours	10	10	10	10	10	10	0	2	2	0	2
192 hours	10	10	10	10	10	10	3	2	1	2	0
436 hours	10	10	10	10	10	10	1	0	0	1	0

Table 5:--Effects of pH on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Test	Number of fish per jar	Number of fish surviving in--											
		Tap water control: pH 6.4 replicates			Tap water control: pH 8.4 replicates			20 ppb of Thiodan: pH 6.4 replicates			20 ppb of Thiodan: pH 8.4 replicates		
		1	2	3	1	2	3	1	2	3	1	2	3
No. 1	10	10	10	10	10	10	10	1	0	0	1	1	1
No. 2	10	10	5	10	10	10	10	0	0	0	1	0	1

Table 6:--Effects of pH 6.4 and 7.4, and solution age on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Number of fish surviving in--											
		Tap water control: pH 6.4 replicates			Tap water control: pH 7.4 replicates			20 ppb of Thiodan: pH 6.4 replicates			20 ppb of Thiodan: pH 7.4 replicates		
		1	2	3	1	2	3	1	2	3	1	2	3
120 hours	10	10	10	10	10	10	10	0	0	1	0	0	1
168 hours	10	10	10	10	10	10	10	6	2	7	0	1	0
240 hours	10	10	9	10	10	10	9	0	0	0	1	4	1

mortalities in toxic solutions at pH 6.4 and 7.4 gave a probability under the null hypothesis of 0.04. This is a low probability in comparison with that calculated above for the effect of calcium and magnesium salts. It suggests some degradation of Thiodan in solutions of pH 6.4; however, survival in these vessels did not appear random, but predominated in those aged for 168 hours. The relatively high survival in two jars may be related to an error in the preparation of solutions, or to an inadvertent selection of resistant fish. In general, I considered the experiment adequate and the original conclusion valid.

The aging of alkaline solutions containing 20 ppb of Thiodan reduces or completely eliminates their toxicity to suckers (tables 7 and 8). Solutions of pH 8.4 were only slightly toxic after 72 hours of aging. The mortalities of fish in solutions aged for 24 and 48 hours averaged 60 and 15 percent respectively. Interpolation of these percentages on the 24-hour concentration-mortality regression gives Thiodan concentrations of about 7.3 and 3.8 ppb and indicates a three- to five-fold degradation of the insecticide.

Nearly all of the toxicant degrades within 24 hours at pH 9.4 (table 8). Mortalities were

Table 7:--Effects of pH 7.4 and 8.4, and solution age on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Number of fish surviving in--				20 ppb of Thiodan: pH 8.4 replicates			
		Tap water control: pH 7.4	Tap water control: pH 8.4	20 ppb of Thiodan: pH 7.4		1	2	3	4
24 hours:									
Test No. 1	10	10	10	1		0	10	0	3
Test No. 2	10	10	10	1		8	3	7	2
48 hours:									
Test No. 3	10	10	10	2		10	10	10	6
Test No. 4	10	10	10	0		8	10	9	6
72 hours:									
Test No. 5	10	10	10	1		10	10	10	10
Test No. 6	10	10	10	1		7	10	10	10
96 hours:									
Test No. 7	10	10	10	2		10	8	10	10
Test No. 8	10	10	10	1		10	10	10	10

Table 8:--Effects of pH 7.4 and 9.4, and solution age on the 24-hour toxicity of 20 ppb of Thiodan to western white suckers at 19° C.

Age of solutions prior to bioassay	Number of fish per jar	Number of fish surviving in--				20 ppb of Thiodan: pH 9.4 replicates			
		Tap water control: pH 7.4	Tap water control: pH 9.4	20 ppb of Thiodan: pH 7.4		1	2	3	4
24 hours:									
Test No. 1	10	10	10	5		10	10	10	10
Test No. 2	10	10	10	2		8	10	10	10
48 hours:									
Test No. 3	10	10	10	3		10	10	8	9
Test No. 4	10	10	10	0		10	10	10	10
72 hours:									
Test No. 5	10	10	10	3		10	10	10	10
Test No. 6	10	10	10	0		10	10	10	8
96 hours:									
Test No. 7	10	10	10	1		10	10	10	10
Test No. 8	10	10	10	0		10	10	10	10

observed in only 4 of 32 potentially toxic solutions and none exceeded 20 percent. All of the control fish survived at pH 7.4 to 9.4 and the mortalities in 20 ppb of Thiodan at pH 7.4 ranged from 50 to 100 percent.

Statistical probabilities for observing certain blocks of data in tables 7 and 8 were

computed under the null hypothesis (table 9). The relative magnitude of the probabilities was considered in assigning significance to the various comparisons. The very low probability that the survival of suckers in Thiodan solutions aged for 24 hours at pH 8.4 was no different from that of fish in the control (comparison 2) indicates residual toxicity. The higher probability

Table 9:--Probabilities, under the null hypothesis, of observing various sets of data taken from experiments on pH.

Comparison	Table number	Age of solutions (hours)	Comparison	Probability
No. 1	7	24	Survival in 20 ppb of Thiodan, pH 7.4, vs. Survival in 20 ppb of Thiodan, pH 8.4	0.0064.
No. 2	7	24	Survival in control, pH 8.4 vs. Survival in 20 ppb of Thiodan, pH 8.4	Approximately 3.78 ⁻⁴⁷ .
No. 3	7	48, 72, 96	Survival in 20 ppb of Thiodan, pH 7.4 vs. Survival in 20 ppb of Thiodan, pH 8.4	Approximately 1.03 ⁻³⁶ .
No. 4	7	48, 72, 96	Survival in control, pH 8.4 vs. Survival in 20 ppb of Thiodan, pH 8.4	0.0254.
No. 5	8	24, 48, 72, 96	Survival in 20 ppb of Thiodan, pH 7.4 vs. Survival in 20 ppb of Thiodan, pH 9.4	Approximately 5.33 ⁻⁵³ .
No. 6	8	24, 48, 72, 96	Survival in control, pH 9.4 vs. Survival in 20 ppb of Thiodan, pH 9.4	0.2069.

for comparison 1, however, suggests that some detoxification of the chemical occurs with 24 hours. The aging of similar solutions for longer periods (comparisons 3 and 4) results in further detoxification which is demonstrated by a reversal in magnitude of probabilities for the respective comparisons. The values for comparisons 5 and 6 indicate extensive detoxification of Thiodan at pH 9.4.

Of the various water-quality factors which were considered as possibly important in the detoxification of Thiodan, alkaline pH is the most significant. Calcium and magnesium ions and mildly acid media appear to have no effect on toxicity. Thus, the residual toxicity of Thiodan can be minimized when treatments are made just prior to anticipated increases in

basicity. We must not discount the interaction of temperature and pH on the residual toxicity of Thiodan. The efficacy of alkaline pH on detoxification of Thiodan at temperatures lower than 19° C. may be considerably less. Berger^{4/} found that 9 days were required to detoxify 10 ppb of Thiodan to rainbow trout at pH 9 and 12° C.

According to Frensch et al. (1959) the fate of Thiodan in water is hydrolysis to Thiodan alcohol. Apparently little is known of the biological toxicity of the alcohol, although it

^{4/} Personal communication from Bernard L. Berger, Chemist, Bureau of Sport Fisheries and Wildlife, Fish Control Laboratory, La Crosse, Wis.

must be relatively nontoxic to fish. Lindquist and Dahm (1957) tested it against one rat, and an oral dose of 1370 mg per kg was not lethal.

Deposition and metabolism of Thiodan

Radioactive Thiodan--The exposure of suckers to 20 ppb of ^{14}C -labeled Thiodan results in the deposition of significant amounts of radioactive substances in their tissues (tables 10, 11, and 12). The mean concentrations of labeled substances in μg per g of dry tissue during 12 hours of exposure are: liver 8.4; gut and feces 5.8; blood 4.4; heart 4.1; gill 3.1; kidney 2.7; gut (empty) 2.7; brain 2.6; skin 1.8; and muscle 1.1.

A concentration of 80 ppb of ^{14}C -labeled Thiodan killed suckers within 2.25 and 9.25 hours of exposure. The mean levels of radioactive compound in their tissues in μg per g of dry tissue are: liver 22.9; gut and feces 14.5; blood 8.9; brain 6.5; kidney 6.0; heart 5.6; gut (empty) 5.3; gill 4.0; skin 2.6; and muscle 1.8 (tables 10, 11, and 12). The livers, guts, blood, brains, and kidneys of fish treated with 80 ppb of ^{14}C -labeled Thiodan contain approximately 2 to 3 times as much radioactive material as was present in the same tissues of fish treated with, but not killed by, a concentration of 20 ppb. The activity levels in several tissues of the former seem highly variable and poorly correlated with exposure. Those in brain, on the other hand, were relatively consistent and may represent the concentration necessary to cause death.

The concentrations of radioactive substances, presumably ^{14}C -labeled Thiodan, in muscles of suckers are relatively low in comparison with those in other tissues. This tissue, however, is probably one of the larger reservoirs for Thiodan deposition because of its relatively greater mass. In order to estimate the total Thiodan deposited in muscle in relation to that added to the external medium, the percent of muscle in the fish and its water content must be considered. Holden (1962) assumed that muscle amounted to 75 percent of the total body weight of brown trout, and Spector (1956)

placed the water concentrations in muscles of several freshwater fish at about 70 percent. Using these percentages, an average weight of fish of 40 grams, and average levels of Thiodan in muscle, I calculated that 9.9 μg of Thiodan were present in the muscle of each fish treated at 20 ppb and 16.2 μg in those killed by 80 ppb. The deposits in muscle comprise 33 and 14 percent respectively of the total ^{14}C -labeled Thiodan added to the external media. A fourfold increase of the Thiodan concentration in the water did not elevate muscle residues by a similar amount.

The small difference between muscle residues of fish treated at 20 or 80 ppb of ^{14}C -labeled Thiodan suggests that the insecticide may reach a level of saturation in muscle lipids. Ludwig, Weis, and Korte (1964) found that aldrin reached a level of saturation in the lipids of rats after which no additional toxicant was stored.

Linear regressions based on the un-averaged tissue concentrations of radioactive substances and exposures were calculated for fish treated at 20 ppb of ^{14}C -labeled Thiodan to estimate correlation and rate of uptake (fig. 2 and table 13). The correlation coefficients for liver, gut and feces, blood, heart, gill, kidney, and brain range from 0.67 to 0.97 and are statistically significant at the 0.05 level of probability. The concentrations of labeled substances in gut (empty), skin, and muscle are not significantly correlated with exposure, particularly those in muscle, which had a coefficient of only 0.29. The tissues containing the greatest concentrations of radioactivity are not necessarily the ones with the highest rates of uptake. For example, gut and feces contain a lower average concentration than liver, but the rate of uptake in the former was approximately 22 percent greater. The intercepts and slopes of the regressions suggest that the uptake of ^{14}C -labeled Thiodan by the various tissues is rapid and probably nonlinear during the first hour of exposure. Thus, the linear regressions cannot be extrapolated linearly to determine uptake rates for the first hour.

Table 10:--Concentrations of radioactive substances in liver, kidney, and gut of western white suckers following various exposures to two concentrations of ^{14}C -labeled Thiodan at 19° C.

Concentration of ^{14}C -labeled Thiodan		Hours Exposure	Number of fish	Radioactive substances ($\mu\text{g/g}$ dry weight of tissue) in					
				Liver		Kidney		Gut	
				Mean	Counting error ^{1/} (\pm)	Mean	Counting error ^{1/} (\pm)	Mean	Counting error ^{1/} (\pm)
20 ppb	1.00	2		4.3	0.4	1.5	0.6	1.2	0.4
20 ppb	2.00	1		4.2	0.4	2.2	0.9	1.8 ^{3/}	0.9
20 ppb	3.00	2		6.3	0.5	2.6	0.5	3.7	0.5
20 ppb	4.00	1		5.0	0.5	1.3	0.5	2.3 ^{3/}	1.1
20 ppb	6.00	2		12.2 ^{2/}	0.4	3.9	0.7	7.2	0.5
20 ppb	8.00	1		10.4	0.8	4.0	1.2	3.8 ^{3/}	1.2
20 ppb	9.00	2		13.9	0.5	2.8	0.4	10.9	0.7
20 ppb	12.00	1		11.1	0.9	4.4	1.2	3.0 ^{3/}	1.1
Overall mean				8.4	0.6	2.7	0.8	5.8	0.3
								2.7 ^{3/}	1.1
80 ppb	2.75 ^{4/}	1		18.8	0.5	4.0	0.6	6.9	0.1
80 ppb	5.50 ^{4/}	2		35.0 ^{2/}	0.9	9.5	0.6	22.1	1.0
80 ppb	9.25 ^{4/}	1		14.8	0.7	4.5	0.5	5.3 ^{3/}	0.8
Overall mean				22.9	0.7	6.0	0.6	14.5	0.6
								5.3 ^{3/}	0.8

^{1/} 0.05 probability limit.^{2/} One sample.^{3/} Gut contents removed.^{4/} Time of death.Table 11:--Concentrations of radioactive substances in blood, heart, and brain of western white suckers following various exposures to two concentrations of ^{14}C -labeled Thiodan at 19° C.

Concentration of ^{14}C -labeled Thiodan		Hours Exposure	Number of fish	Radioactive substances ($\mu\text{g/g}$ dry weight of tissue) in					
						Heart		Brain	
				Mean	Counting error ^{1/} (\pm)	Mean	Counting error ^{1/} (\pm)	Mean	Counting error ^{1/} (\pm)
20 ppb	1.00	2		2.1 ^{2/}	0.2	2.2	0.7	1.5	0.4
20 ppb	2.00	1		3.5	0.9	4.5	1.6	2.3	0.7
20 ppb	3.00	2		---	---	2.6	0.8	1.8	0.4
20 ppb	4.00	1		3.9	0.7	2.5	1.7	1.6	0.6
20 ppb	6.00	2		---	---	4.2	0.9	3.5	0.4
20 ppb	8.00	1		5.3	1.7	5.3	1.7	2.8	0.7
20 ppb	9.00	2		---	---	4.0	0.7	3.4	0.4
20 ppb	12.00	1		7.0	0.9	9.7	4.0	3.7	0.1
Overall mean				4.0	0.9	4.1	1.5	2.6	0.6
80 ppb	2.25 ^{3/}	1		---	---	5.9	0.6	6.2	0.4
80 ppb	5.50 ^{3/}	2		11.1	0.1	7.3	1.1	7.3	0.6
80 ppb	9.25 ^{3/}	1		6.7	0.4	3.7	1.2	5.0	0.7
Overall mean				8.9	0.3	5.6	1.0	6.5	0.6

^{1/} 0.05 probability limit. ^{2/} One sample. ^{3/} Time of death.

Table 12:--Concentrations of radioactive substances in skin, gill and muscle of western white suckers following various exposures to two concentrations of ^{14}C -labeled Thiodan at 19° C.

Concentration of			Radioactive substances ($\mu\text{g/g}$ dry weight of tissue) in					
^{14}C -labeled Thiodan	Hours Exposure	Number of fish	Skin		Gill		Muscle	
			Mean	Counting error ^{1/} (\pm)	Mean	Counting error ^{1/} (\pm)	Mean	Counting error ^{1/} (\pm)
20 ppb	1.00	2	1.1	0.8	1.9	0.5	1.2	0.3
20 ppb	2.00	1	0.6	0.7	2.7	1.6	0.5	0.2
20 ppb	3.00	2	2.5	1.1	3.7	1.7	0.8	0.2
20 ppb	4.00	1	1.1	1.1	3.1	1.5	0.6	0.2
20 ppb	6.00	2	2.4	0.9	2.8	1.1	1.8	0.3
20 ppb	8.00	1	1.2	0.5	2.4	0.5	1.3	0.4
20 ppb	9.00	2	1.3	0.8	3.2	0.9	1.1	0.3
20 ppb	12.00	1	4.9	2.9	6.4	1.7	1.3	0.4
Overall mean			1.8	1.1	3.1	1.2	1.1	0.3
80 ppb	2.25 ^{2/}	1	3.1	0.9	4.1	0.4	1.4	0.2
80 ppb	5.50 ^{2/}	2	3.3	0.4	4.9	0.7	3.3	0.3
80 ppb	9.25 ^{2/}	1	1.3	1.1	3.1	0.5	0.8	0.2
Overall mean			2.6	0.8	4.0	0.5	1.8	0.2

1/ 0.05 probability limit.

2/ Time of death.

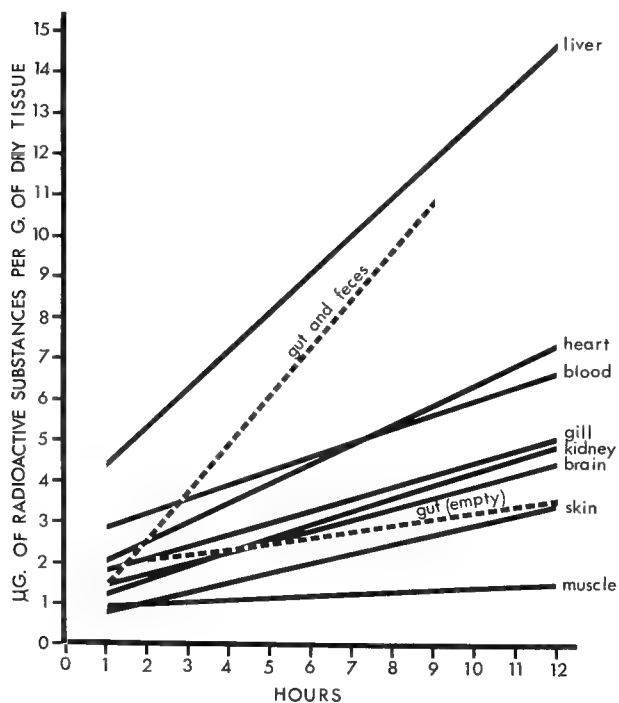


Figure 2:--Uptake of radioactive substances in tissues of western white suckers during their exposure to 20 ppb of ^{14}C -labeled Thiodan at 19° C.

Table 13:--Slopes and correlation coefficients for regressions in figure 2.

Tissue	Slope (b)	Correlation coefficient (r)
Liver	0.93	0.85
Gut and feces	1.18	0.97
Heart	0.48	0.78
Blood	0.34	0.89
Gill	0.29	0.67
Kidney	0.33	0.84
Brain	0.25	0.82
Gut (empty)	0.14	0.66
Skin	0.23	0.48
Muscle	0.05	0.29

In general, with the exception of gut and feces, the higher concentrations and rates of uptake of labeled chemical occur in blood and those tissues containing relatively large amounts of blood. The actual volumes of blood in sucker tissues were not measured; however, Hoffert (1966) found that livers, gills, kidneys, and muscles of lake trout contained 17.0, 16.5, 8.9, and 0.4 percent blood respectively. If

similar amounts of blood are present in sucker tissues, and if the concentrations of radioactive substances were wholly dependent on blood content in tissue, then one would expect to detect concentrations of radioactivity in tissues which vary according to blood content, and the radioactivity in blood. The regressions in figure 2 indicate that radioactive substances in liver, gill, kidney, and muscle were not sufficiently lower than those in blood to be correlated entirely with the blood content of the tissues. Since the ranking for concentrations of radioactivity in gill, kidney, and muscle was similar to Hoffert's blood values, the blood content may have some influence on the level of radioactivity in the tissue.

The observation that the levels of labeled substances in the liver were much greater than those in blood suggests that Thiodan is removed from the blood by this organ and stored. The lower deposition and uptake in other tissues, except gut and feces, may be due to this function of the liver.

The removal of the contents of the gut lowers the concentration of radioactivity in the gut (table 10 and fig. 2). This demonstrates that the majority of the radioactivity present in the intestine is contained in the feces rather than in the tissue itself. Mount (1962) treated bluntnose minnows with endrin and detected it within the gut. In his opinion, endrin may have entered the tract along with ingested water. Because few fresh water teleosts drink significant amounts of water (Black, 1957), another avenue for the entrance of Thiodan into the intestinal tracts of suckers was sought. Since the samples of gut used in the analyses were collected posterior to the entrance of the gallbladder, I postulated that the radioactivity may have entered with the bile.

A second series of experiments was necessary to test the hypothesis that Thiodan is excreted in bile. This also provided an opportunity to evaluate the solubility characteristics of the labeled materials in tissues and bile of ^{14}C -labeled Thiodan treated fish. Northern

creek chubs and western white suckers were exposed to 40 ppb of ^{14}C -labeled Thiodan for 3 or 5 hours at 21° C. The concentrations and relative benzene-water solubilities of radioactive substances in the bile, and in chub tissues are shown in tables 14 and 15. Simple, ethanolic extracts of chub tissues removed approximately 62 to 100 percent of the radioactivity (table 14). The majority of the labeled substances in the lipid extracts are separated, after benzene-water partition, into the benzene fraction. The remainder, ranging from 9 to 20 percent of the total, appear water soluble; however, a portion or all of these may have been benzene soluble since emulsions were included with this fraction.

The concentrations of benzene-soluble radioactive substances are highest in liver, brain, kidney, and blood, in that order (table 14). Extension of the exposure from 3 to 5 hours did not increase the amounts deposited in the tissues.

Conclusions about distribution of Thiodan based on measurements of radioactivity must be qualified because of the possible metabolic destruction of the chemical. The fact that the radioactive substances extracted from chub tissues were soluble in ethanol and benzene indicates that activity levels in chubs and those measured earlier in suckers, represent deposits of Thiodan, or at least the aromatic portion of the molecule. According to Hawk, Oser, and Summerson (1954), aromatic compounds are fairly resistant to oxidation, whereas their acyclic side chains may be catabolized.

Radioactive materials are present in the bile of chubs and suckers which are treated with ^{14}C -labeled Thiodan (table 15). Ninety-four to 100 percent of the activity is separated, by benzene-water partition, into the water fraction. The concentrations in this fraction range from 88 to 158 μg per g of dried bile for chub and 36.5 to 48.0 μg per g for sucker. Semiquantitative measurements were made of the water-soluble metabolite in sucker bile. Incubation of the bile with β -glucuronidase converted the labeled metabolite to a benzene-

Table 14:--Benzene- and water-soluble radioactive substances in extracts of tissues of northern creek chubs exposed to 40 ppb of ^{14}C -labeled Thiodan at 21° C.

Radioactive substances ($\mu\text{g/g}$ dry wt. of tissue) in										
Tissue and exposure	Number of fish	Benzene soluble fraction		Water soluble fraction		Extracted tissue		Percent in benzene	Percent in water	Percent in tissue
		Counting error ^{1/}		Counting error ^{1/}		Counting error ^{1/}				
		Mean	(\pm)	Mean	(\pm)	Mean	(\pm)			
Liver:										
3 hours	2	8.2	0.4	1.0	0.2	1.7	0.4	75.0	9.0	15.6
5 hours	2	4.5	0.2	0.7	0.2	1.8	0.3	64.0	10.0	25.7
Kidney:										
3 hours	2	3.3	0.5	0.9	0.4	0.3	0.3	73.3	20.0	6.7
5 hours	2	4.2	0.8	0	-	2.6	0.6	61.8	0	38.2
Blood:										
3 hours	2	2.3	0.3	0.6	0.3	0.4	0.2	69.7	18.2	12.1
5 hours	1	2.0	0.5	0.4	0.3	0	-	83.3	16.7	0
Brain:										
3 hours	2	5.3	0.6	0	-	0	-	100	0	0
5 hours	2	3.1	0.6	0	-	0	-	100	0	0

^{1/} 0.05 probability limitTable 15:--Benzene- and water-soluble radioactive substances in gallbladder bile of two species of fish exposed to 40 ppb of ^{14}C -labeled Thiodan, and the effect of beta-glucuronidase on substance solubility.

Species and fish number		Before incubation of bile with enzyme; radioactive substances ($\mu\text{g/g}$ of dry bile) in---						After incubation of bile with enzyme; radioactive substances ($\mu\text{g/g}$ of dry bile) in---					
		Benzene soluble fraction			Water soluble fraction			Benzene soluble fraction			Water soluble fraction		
		Con- tra- tion	Counting error ^{1/} (\pm)	Per- cent	Con- tra- tion	Counting error ^{1/} (\pm)	Per- cent	Con- tra- tion	Counting error ^{1/} (\pm)	Per- cent	Con- tra- tion	Counting error ^{1/} (\pm)	Per- cent
Northern creek chub:													
No. 1	3	5.6	0.9	6.0	88.0	5.5	94.0	-	-	-	-	-	-
No. 2	3	0.0	-	0.0	158.0	5.8	100.0	-	-	-	-	-	-
No. 3	5	4.5	0.9	3.5	124.0	5.9	96.5	-	-	-	-	-	-
Western white sucker:													
No. 4	5	1.5	0.5	3.9	36.5	1.7	96.1	46.0	1.7	99.4	0.3	0.6	0.6
No. 5	5	2.2	0.5	4.4	48.0	1.7	95.6	41.4	1.0	97.2	0.1	0.6	2.8

^{1/} 0.05 probability limit.

soluble form. Thus, the results suggest that the liver removes Thiodan from the blood stream, changes it to an aromatic metabolite, and conjugates it with glucuronic acid. Then the conjugate is discharged with bile into the

gallbladder. The subsequent release of bile containing the radioactive conjugate into the gut may explain the high levels of radioactivity present in feces of suckers (fig. 2).

Metabolism and excretion of Thiodan in fish appears to be similar to that observed in rats and rabbits for the excretion of other chlorinated insecticides, such as ^{14}C -labeled aldrin and ^{14}C -labeled dieldrin. These compounds are metabolized by the liver into more hydrophilic compounds and excreted via the bile into the intestinal canal (Korte, Ludwig, and Vogel, 1962; and Morsdorf et al., 1963).

Ludwig et al. (1964) found that rats fed low doses of ^{14}C -labeled aldrin over long periods excreted aldrin, dieldrin, and unidentified hydrophilic products in both feces and urine. The total activity excreted daily over 12 weeks increased from approximately 50 to 100 percent of the daily dose. The amount excreted in the feces was 10 to 20 times as great as that in urine. The hydrophilic compounds in feces and urine were not alike chromatographically, and alkaline hydrolysis of the urinary metabolites gave a compound with acidic properties. Aromatic acids such as benzoic acid are conjugated with glucuronic acid (Long, 1961).

Additional evidence supporting the proposed pathway for the metabolism and excretion of Thiodan by fish will be discussed in the next section.

Nonradioactive Thiodan--As stated earlier, further trials with radioactive Thiodan were impossible owing to its degradation to what appeared to be Thiodan alcohol. To strengthen the findings of the radiation experiments, additional fish were treated with technical Thiodan, and its residues or metabolites in tissues and bile were analyzed chemically.

In preliminary experiments, goldfish and carp were killed with 20 ppb of Thiodan and their tissues analyzed. Traces of the insecticide were found in muscle, but the levels were beyond the range of accurate measurement by the analytical method. The possibility that concentrations of Thiodan in the tissues could be elevated by exposing the fish for longer periods to lower concentrations was tested by treating one goldfish and one carp daily with 7.0 ppb.

The carp died after the second treatment and the goldfish after ten. Only traces of the toxicant were found in carp, but 1.1 μg per g wet weight were measured in muscle of the goldfish and 0.5 μg per g in its gills. Seven μg per ml were found in the bile after incubation with beta-glucuronidase. Since only traces of Thiodan were found in other goldfish tissues, samples from a number of fish were combined in later trials in order to measure Thiodan in these tissues satisfactorily. Repeated treatments of goldfish with ordinarily subacute concentrations apparently cause mortality and induce measurable residues of Thiodan.

Ten goldfish weighing 96 to 337 grams were exposed daily and individually to a concentration of 7.0 ppb of Thiodan. The order in which the fish died according to their weight, sex, length of exposure, and total Thiodan treatment is shown in table 16. The two smallest specimens were males, and they succumbed on the fourth and fifth days of treatment after exposure to combined concentrations of 28 and 35 ppb of Thiodan respectively. The remaining fish were females which died at intervals up to 13 days after the initial exposure. Two of the largest individuals survived 20 applications, or exposure to a total of 2.10 mg of Thiodan.

The largest deposits of Thiodan residues in goldfish tissues ranged from 3.40 to 12.76 μg per g of wet tissue in the bile, liver, brain, and abdominal fat respectively (table 16). The mean residues in μg per g of wet tissue for other tissues are: scales and fins 0.45; gonad 0.67; gill 0.86; heart, kidney, spleen, and blood 1.39; muscle 1.67; and skin 2.48. Thiodan was not detected in the gut and feces.

Susceptibility of goldfish to repeated subacute concentrations of Thiodan is strongly related to their weight. The lightest individuals are the most susceptible. The correlation of weight of fish with number of treatments before their death gives a coefficient of 0.89.

The correlation of weight with number of treatments suggests an accumulation of

Table 16:--Residues of Thiodan in goldfish treated daily in fresh solutions containing 7 ppb of Thiodan at 12° C.

Fish	Weight	Sex	Thiodan treatments		Concentration of Thiodan in µg per g wet weight in---									
			Number before death	Amount before death (mg)(ppb)	Mus-	Bile ^{1/}	Gill	Scales	con-	Gonad	Heart kidney spleen	Brain ^{2/}	Peri-to-neal ^{3/}	fat ^{3/}
No. 1	109	M	4	0.42 28	1.41									---
No. 2	96	M	5	0.53 35	2.54	0	Samples composed of tissues of fish 1 to 5							---
No. 3	129	F	7	0.74 49	2.10		0.69	3.28	0.77	5.61	0	0.73	1.23	---
No. 4	142	F	8	0.84 56	1.15									---
No. 5	188	F	11	1.16 77	----									---
No. 6	191	F	13	1.37 91	1.99	3.67	Samples composed of tissues of fish 6 to 10							---
No. 7	184	F	13	1.37 91	2.13							8.55		---
No. 8	277	F	13	1.37 91	0.95	3.40	1.03	1.69	0.21	5.32	0	0.61	1.54	---
No. 9	241 ^{4/}	F	20	2.10 140	1.09	----								12.76
No. 10	338 ^{4/}	F	20	2.10 140	----									
Mean					1.67	3.53	0.86	2.48	0.45	5.47	0	0.67	1.39	8.55 12.76

1/ Samples composed of bile of two fish. Bile incubated with beta-glucuronidase. Mean based on positive values only.

2/ Sample composed of brains of 10 fish.

3/ Sample composed of fat from fish 9 and 10.

4/ Alive after 20 treatments.

1.2, 3.5, 2.1, 3.4, 6.7, 3.0, 5.6, 3.7, 6.8, and 6.1 percent. In terms of dry weight, most of the values are between 14 and 26 percent. The correlation of lipid content with the number of treatments which they survived gives a coefficient of 0.77 indicating that individuals with more muscle lipids survive longest.

The male goldfish were the most susceptible to Thiodan, but also they were the smallest specimens. These two variables were confounded, and the independent influence of sex could not be evaluated.

Thiodan with time; larger fish, because of greater mass, require a longer exposure to attain the same concentration of Thiodan in their tissues as smaller fish. Residues in fish treated for up to 20 days are essentially no greater than those in individuals receiving up to 11 treatments. The coefficient of correlation for residues in muscle, based on wet weight, with days of exposure is 0.38.

The differences in sizes of the goldfish are due primarily to weight rather than length. The heaviest fish weighed approximately three times as much as the smallest but was only 30 percent longer. This length-weight relation suggests that the heavier and more resistant individuals also contained the greatest amount of lipids. The lipids in goldfish muscle were calculated as a percent of the wet weight of the tissue and for fish 1 to 10 (table 16), are:

The micrograms of Thiodan per gram of muscle lipids extracted from each of the 10 fish are, in order: 113.28, 71.93, 97.73, 33.68, 64.37, 37.92, 25.39, and 16.15. The concentrations are negatively correlated with the number of treatments, weight of fish, and the lipid content of the muscle. The coefficients are 0.78, 0.75, and 0.85 respectively. Whereas the quantity of the Thiodan in muscle was relatively constant, an increase in lipid content

allows a decline of Thiodan in muscle lipids of heavier fish. These data indicate that muscle lipids aid in the "detoxication" of Thiodan by providing a reservoir for its storage and dilution. However, since all but the two most resistant goldfish died during the experiment the actual amount of poison in muscle appears to be more closely allied with mortality than its concentration in lipids. Thus, a physically greater mass of lipids in heavier fish contribute more to their survival than the degree of saturation of lipids with Thiodan. The high concentration of Thiodan in abdominal fat of fish 9 and 10 (table 16) indicates that insecticide storage in this tissue may also contribute to the resistance of the heavier individuals.

Thiodan concentrations are considerably greater in brain than those in gill, liver, and a composite of heart, kidney, spleen, and blood (table 16). This is contrary to observations made on suckers where the high concentrations of what was presumed to be Thiodan were associated with blood containing tissues. Also, the skin of suckers contained less Thiodan than that of goldfish. This apparent inconsistency may be related to a greater lipid content of goldfish, and their longer exposure to Thiodan.

Thiodan is adsorbed on or absorbed into the scales and fins of goldfish (table 16). Although the levels are comparatively low, they demonstrate an ability of the body surfaces to remove and concentrate the insecticide from the external medium. Holden (1962) found that DDT was concentrated by the external mucus of brown trout, and he pointed out the possibility of its entry into fish through the skin.

The gonads of suckers treated with ^{14}C -labeled Thiodan were not assayed for radioactivity, but composite samples of goldfish gonads contain 0.67 to 0.73 μg of Thiodan per g of wet tissue (table 16). In toxicity trials, the fertilized eggs of rainbow trout were extremely resistant to Thiodan; however, incorporation of the insecticide into fish eggs before spawning may have an adverse effect on their fertility or survival. Residues of chlorinated insecticides such as DDT in fish eggs are

suspected of reducing their reproductive success (Burdick et al., 1964; Cuerrier, Keith, and Stone, 1967; and Macek, 1968).

The water content of samples of goldfish muscle ranged from 71.3 to 78.1 percent and averaged 75.2 percent. The two fish receiving 20 treatments with Thiodan (table 16) had the lowest water concentrations in their muscles. In general, however, there appeared to be no relation between the mortality of fish and tissue water.

The measurement of Thiodan in various tissues of goldfish by relatively specific chemical methods supports an earlier belief that the majority of the radioactive substances detected in suckers were indeed ^{14}C -labeled Thiodan. According to Barnes and Ware (1965) technical Thiodan contains four components, the high and low melting point isomers, Thiodan alcohol, and Thiodan ether. The chemical method used in my investigation measures both isomers and the Thiodan alcohol but not the ether. The low melting point isomer and the alcohol are not recoverable from the Florisil-carbon column. The possibility that a small portion of the radioactivity in suckers did not represent Thiodan is suggested by the incomplete extractions of radioactive substances from some chub tissues with ethanol (table 14). The remaining activity may possibly have been associated with water-soluble metabolites.

Thiodan is not detected colorimetrically in petroleum ether extracts of goldfish bile until after the samples are incubated with beta-glucuronidase, nor in extracts of gut and content (table 16). This was expected considering the existence of water-soluble radioactive substances found in the bile of suckers and chubs (table 15). The wavelength of maximum absorption for the colored product is 534 $\text{m}\mu$, the exact wavelength used for measurements of Thiodan. Its average concentration in bile is 3.5 μg per ml, after applying a correction for a background of unidentified constituents of the bile. The uncorrected concentrations agree closely with that measured in bile during the preliminary investigation. The average

background was 3.6 μg per ml. It was derived by measuring the absorbance, at 534 m μ , of two samples which apparently contained no Thiodan; one made up of bile from fish 1 and 2 and another from fish 3 and 4. In these samples, peaks of maximum light absorption were not detected at 534 m μ .

The apparent lack of Thiodan metabolites in the bile of some goldfish may be attributed to: amounts below the limits of detectability due to small volumes of bile or slow rates of conjugate formation; or inhibition of beta-glucuronidase. The volumes of bile in fish 1 to 4 were 0.4 to 0.5 ml whereas those in the remaining fish usually exceeded 0.7 ml. Ludwig et al. (1964) found that rats excreted greater amounts of aldrin metabolites the longer they were maintained on an aldrin containing diet. Beta-glucuronidase is inhibited by several substances including a product of the hydrolysis, glucuronic acid (Long, 1961).

The hydrolysis of water-soluble metabolites of Thiodan, in experiments with both suckers and goldfish, with beta-glucuronidase indicates that these species are able to metabolize the insecticide and conjugate it with glucuronic acid. According to Harper (1959), in mammals uridine diphosphate glucuronic acid (UDPGA) is the active form of the acid involved in the conjugation of chemicals containing carboxyl and hydroxyl groups. It is formed by the oxidation of UDP-glucose which is catalyzed by a diphosphopyridine nucleotide-dependent UDPG dehydrogenase. The conjugation reaction requires glucuronyl transferase. My results imply that not only are both enzymes present in the livers of these species, but also that this organ is able to convert the insecticide to a substance containing carboxyl or hydroxyl groups. The conjugable substance is probably Thiodan alcohol since it, like Thiodan, reacts colorimetrically.

Barnes and Ware (1965) studied the metabolism of Thiodan in house flies. It was oxidized to Thiodan sulfate, a slightly less toxic compound which was considered to be an intermediate in the detoxication process.

Their analyses of feces revealed water- and acetone-soluble substances which they believed were glucoside, or glucosiduronic conjugates of Thiodan metabolites. Although they did not establish the identity of the conjugate, they did eliminate the possibility of conjugation at the terminal sulfur of Thiodan. Thus, experiments with fish and house flies suggest at least one pathway for the biochemical degradation of Thiodan. First, oxidation to Thiodan sulfate, then conversion to Thiodan alcohol, followed by conjugation with glucuronic acid and excretion in the feces. The results of Barnes and Ware (1965) also indicate that the resistance of flies to Thiodan may be controlled by the rate of its oxidation, or the rate of conjugation of the metabolite.

The lipids of fish may serve as a reservoir for depositing the quantities of Thiodan which exceed those that can be immediately and effectively detoxified in the degradation pathway. Then, the deposits may be released and detoxified gradually during the turnover of lipids. Ludwig et al. (1964) reported that rats which were given daily sublethal doses of aldrin in their food were eventually able to excrete the total daily dose as hydrophilic metabolites. After the last dose, the rate of metabolite excretion declined slowly as did the concentration of aldrin in the fat.

In the trials with goldfish, Thiodan may have been released from muscle lipids and its toxicity added to that of later treatments because the fish were fasted throughout the tests. The replenishment of muscle and liver glycogen in fish is dependent on food or conversion from protein and fat (Black et al., 1960; Change and Idler, 1960), and it is conceivable that Thiodan was liberated during fat catabolism. Graham (1960) believed that poor condition and physiological stress of fish may result in stores of DDT becoming lethal.

Another nutritional aspect to be considered in the toxicity of Thiodan to fish is the systemic level of precursors available for the formation of glucuronic acid. As mentioned earlier, glucose is the forerunner of glucuronic

acid in the uronic acid pathway. Therefore, deficiencies in glucose or glycogen might retard the conjugation of Thiodan metabolites because of insufficient glucuronic acid. Brodie and Maickel (1962) found that frogs which were pre-fed glucose converted phenols largely to glucosiduronic acids. Without glucose, the phenols were changed primarily to ethereal sulfates.

Although conjugation reactions involving glucuronic acid are well known in mammals (Harper, 1959), there is conflicting evidence in the literature regarding the abilities of fish to form glucosiduronic acids with other compounds. Brodie and Maickel (1962) reported that livers of several species, including goldfish, do not conjugate phenols with glucuronic acid *in vitro*, or, *in vivo* after intraperitoneal injections of phenol. They found that liver microsomes contained glucuronyl transferase, and phenyl glucuronids were formed when UDPGA was added to liver preparations. However, the microsomes apparently lack UDPG-dehydrogenase for conversion of UDPG to UDPGA. On the other hand, Grajcer and Idler (1963) measured conjugates of testosterone in the blood and testes of sockeye salmon. The hormone was released by the action of β -glucuronidase, and they concluded that it had been conjugated with glucuronic acid.

The results of my investigations and those of Grajcer and Idler (1963) do not support the conclusions which Brodie and Maickel (1962) have drawn concerning the inability of fish to form glucosiduronic acids. Certainly, further research is indicated to resolve these apparently contradictory findings. Perhaps tissues other than the liver, such as the kidney, are also involved in the conjugation and excretion of Thiodan metabolites in fish.

SUMMARY

The median tolerance limits (TLm) of rainbow trout and western white suckers to Thiodan are 0.3 to 8.1 ppb, depending on temperature and exposure. *Daphnia* and damselfly naiads are at least seven times as resistant

than fish. The naiads, in contrast to other species, appear more susceptible at colder temperatures. The effect of temperature on the toxicity of Thiodan to fish and invertebrates diminishes with exposure. Thiodan is nontoxic to trout eggs, but after hatching, the fish become increasingly susceptible with age.

The toxicity of Thiodan to suckers is not influenced by calcium or magnesium salts in the water or by mildly acidic conditions. A pH of 8.4 or 9.4 reduces or eliminates the toxicity of the insecticide within 24 to 96 hours at 19° C. Degradation at these pH's is slower at cooler temperatures.

Investigations with carbon ^{14}C -labeled Thiodan indicate that the insecticide is taken up and deposited in various tissues of fish. The uptake is highest in liver and in the gut and feces. Rates of uptake are slower in heart, blood, gill, kidney, and brain. Western white suckers exposed to 20 ppb of labeled compound contained mean concentrations in the tissues of 1.1 to 8.4 μg per g of dry tissue. The residues in muscle and skin correlate poorly with exposure.

Goldfish are killed by daily exposures to fresh solutions containing 7 ppb of Thiodan. They succumb according to their size and the lipid content of muscle. Residues of 0.95 to 2.54 μg per g of wet tissue are detected colorimetrically in muscle. They correlate poorly with the number of treatments. Lesser amounts are found in the gonad, gill, heart, kidney, spleen, and blood, and greater levels are found in skin, liver, brain, and peritoneal fat.

Radio-tracer and chemical techniques reveal a water-soluble metabolite of Thiodan in the bile of western white suckers, northern creek chubs, and goldfish. Analyses suggest that Thiodan is degraded metabolically to its alcohol which is then conjugated with glucuronic acid and excreted via the bile into the feces.

In general, Thiodan appears useless as a selective piscicide against so-called trash

species. The chemical may be useful, however, where selectivity among fish is unimportant because: it is highly toxic to fish and less toxic to many aquatic invertebrates; it is less toxic to mammals than several other chlorinated insecticides; and it does not persist long in water of relatively high pH and temperature. The exposure of fish to Thiodan probably renders them unfit for human consumption since residues occur in edible tissues such as the muscle.

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**36. A Method for Rating Chemicals
for Potency Against Fish
and Other Organisms**

**DIVISION OF FISHES
U.S. NATIONAL MUSEUM**

**37. Comparative Toxicity of
29 Nitrosalicylanilides
and Related Compounds
to Eight Species of Fish**

**38. Toxicity of 33NCS to
Freshwater Fish and Sea Lampreys**



**United States Department of the Interior
Fish and Wildlife Service
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A METHOD FOR RATING CHEMICALS FOR POTENCY AGAINST FISH AND OTHER ORGANISMS

By Leif L. Marking, Chemist
Bureau of Sport Fisheries and Wildlife
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ABSTRACT.--A potency rating is presented by which the toxicity of chemicals to organisms can be assessed with a minimum of data from preliminary bioassays. This method permits effective and rapid evaluation of toxicity when data from preliminary tests are inadequate for statistical analysis. In bioassays of chemicals against fish, the mortality is recorded in 3- to 96-hour tests when exposed to three concentrations. The potency rating of a bioassayed chemical, when compared with the maximum value on the rating scale, indicates the relative toxicity of the chemical. Concentrations and exposure times can be changed to fit a specific bioassay program. Results of 24- or 48-hour bioassays can be compared with 96-hour bioassays because the maximum values of the rating scales are approximately equal at all time intervals. The potency rating is more applicable to toxicants which have similar dose-effect curves.

Data from preliminary bioassays are usually tedious and difficult to analyze since the toxicity is not defined by statistical parameters such as those described by Litchfield and Wilcoxon (1949). A minimum number of organisms and concentrations limit statistical evaluations for median tolerance limits. Preliminary bioassay data usually indicate toxic ranges of concentrations and are followed by more definitive tests. However, a greater number of chemicals can be tested in preliminary trials such as those described by Lennon and Walker (1964), but a mathematical method is needed for analyzing and summarizing these data in a form that is easily stored and retrieved. In programs in which many chemicals are not tested beyond preliminary screening, the potency rating of a large number of compounds is useful in making comparisons and selecting those having potential. It also reduces the bulk of data to easily comprehended terms.

POTENCY RATING

Potency is necessarily stated in absolute units for therapeutic applications, but for comparison of drugs relative potency is a more convenient term, as Fingl and Woodbury (1965) point out. They also state that dose-effect curves which are similar in slope permit comparisons of relative potencies, while those with dissimilar dose-effect curves do not. Relative potency is more applicable to bioassay data derived from related chemical structures or from chemicals producing similar biological effects on the test organisms.

Relative potency is described by Finney (1962) and is calculated as a constant difference between the dosage-response regressions of two stimuli. This method does not apply to preliminary type data which cannot be analyzed by probit regression methods.

I have therefore derived an equation expressing relative activities of chemicals to fish or other organisms. This equation defines potency rating.

The potency rating effectively separates toxic from nontoxic chemicals. It also indicates the degree of general toxicity and the differential toxicity among species tested. The values obtained are compared with a maximum potency rating calculated by assuming complete mortality at all concentrations and time intervals.

Preliminary screening yields data on numbers of organisms killed at selected concentrations in multiples of 10 that are easily analyzed by comparing their potency ratings. Mortality is dependent upon the concentration and exposure time. Potency rating (Pr) is dependent on the mortality at each concentration and each exposure time. In addition, the number of tests must be considered, since Pr is the accumulation of values throughout a bioassay period. The relation of Pr to the variables involved is suggested by formula 1 where mortality is a function of concentration and time (table 1). In essence, the mortality (M) is multiplied by the reciprocal of the concentration (C) times the reciprocal of the log of time (T) at each concentration and time

interval. The sum of these factors is divided by the log of the number of observations (N). The arrangement of the values used in deriving this index of activity does not follow any particular mathematical relations, and the values do not express units of measurement. The Pr merely indicates the activity of one test substance in comparison with others.

Reciprocals for concentration and the log of time are necessary to emphasize the effects of lower concentrations and shorter exposure periods (figs. 1 and 2). If the reciprocal relations are neglected, the accumulated potency rating increases drastically in longer exposures even though none of the organisms die during this period, and the effects of higher concentrations are weighted more heavily. The effects of the reciprocals are best seen in table 2, where maximum potencies (Pmax) are calculated for five time intervals and three concentrations. I have assumed that 10 organisms die among 10 tested in each case. The contributions of potency ratings are listed at each time interval under each concentration of 0.1, 1.0, and 10 ppm. Pmax contribution at 3 hours at 0.1 ppm is 209.60, whereas that for 96 hours at 0.1 ppm is 50.45, and subsequent contributions at higher concentrations are less by factors 0.1 and 0.01. Therefore, chemicals which are

TABLE 1.--Formulas for potency rating

$$(1) \quad Pr = \frac{\sum_{j=1}^5 \sum_{i=1}^3 \frac{M(C_i, T_j)}{C_i \log T_j}}{\log N}$$

Where M = mortality among the total number tested

C = concentration

T = time of exposures in hours, $T > 1$

N = number of observations, $N > 1$

j = finite variables of T: $j_1 = 3, j_2 = 6, j_3 = 24, j_4 = 48, j_5 = 96$

i = finite variables of C: $i_1 = 0.1, i_2 = 1.0, i_3 = 10$

When the individual concentrations are considered, formula 1 expands to:

$$(2) \quad Pr = \frac{\sum_{j=1}^5 \left[\left(M \times \frac{1}{0.1} \times \frac{1}{\log T_j} \right) + \left(M \times \frac{1}{1.0} \times \frac{1}{\log T_j} \right) + \left(M \times \frac{1}{10} \times \frac{1}{\log T_j} \right) \right]}{\log N}$$

When the observation times are considered also, the formula expands further to:

$$(3) \quad Pr = \frac{\left(M \times \frac{1}{0.1} \times \frac{1}{\log 3} \right) + \left(M \times \frac{1}{1.0} \times \frac{1}{\log 3} \right) + \left(M \times \frac{1}{10} \times \frac{1}{\log 3} \right) + \dots + \left(M \times \frac{1}{10} \times \frac{1}{\log 96} \right)}{\log N}$$

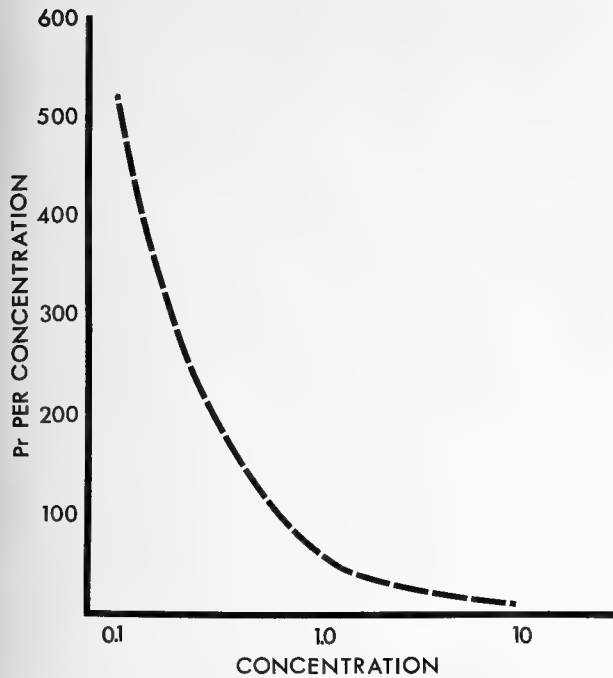


Figure 1.--The potency rating is larger at lower concentrations through the reciprocal relationship.

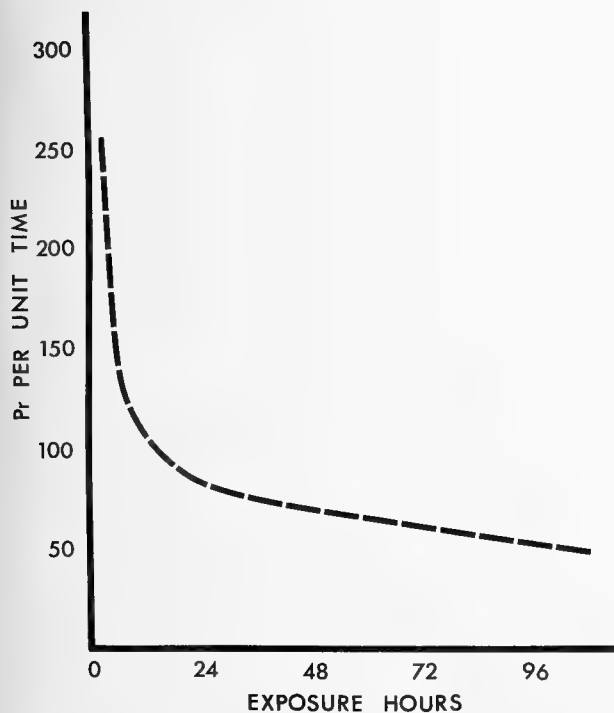


Figure 2.--The potency rating is larger at shorter exposure periods through the reciprocal relationship.

TABLE 2.--Maximum potencies at three concentrations and five time intervals

Exposure	log T	$\frac{1}{\log T}$	N	log N	Concentration (ppm)			Sum at T	Pmax
					0.1	1.0	10.0		
3 hours...	0.4771	2.0960	3	0.4771	209.60	20.96	2.10	232.66	487.65
6 hours...	0.7782	1.2850	6	0.7782	128.50	12.85	1.29	142.64	482.27
24 hours...	1.3802	0.7245	9	0.9542	72.45	7.25	0.72	80.42	467.11
48 hours...	1.6812	0.5948	12	1.0792	59.48	5.95	0.59	66.02	483.45
96 hours...	1.9823	0.5045	15	1.1761	50.45	5.04	0.50	55.99	491.23
Total....	--	--	--	--	520.48	52.05	5.20	577.73	--

highly toxic in short exposure periods indicate greater potency than chemicals which are effective only in longer exposures. Chemicals which are toxic only at higher concentrations will definitely yield lower potency ratings regardless of exposure time. The values at 1.0 and 10 ppm are rounded off to two decimal places.

The divisor in the formula, log N, accounts for the number of observations being analyzed. This arbitrary factor also reduces the additive effect of potency once the fish are recorded dead. Pmax in table 2 indicates little change at any time interval, and data from 24- or 48-hour tests could be compared with data from 96-hour tests. The maximum potency in a 96-hour bioassay is 491.23; thus it becomes the standard for comparison of the calculated experimental potencies. If the potencies were merely summed at the various time intervals, the maximum potency would be 577.73 (table 2), but these effects are cumulative, and the sum depends on the number of time intervals in the testing program. This cumulative potency increases even though no more fish die.

The size of the number describing Pr and the number of digits the Pr contains adequately indicate the toxicity with respect to time. Since Pmax is 467.11 to 491.23 depending on T, and the contribution at 0.1 ppm is a 3-digit whole number, I have assumed that 3-digit whole-number Pr's indicate mortality at 0.1 ppm. Corresponding, 2-digit whole numbers indicate mortality at 1.0 ppm (maximum contribution = 52.05) and values less than 10 indicate mortality at 10 ppm (maximum contribution = 5.20). If none of the specimens die at the highest concentration, the value becomes zero. The Pr values can vary from

0 to 491.23, and their proportion of the maximum potency with respect to T defines the relative activity. Values greater than 300 indicate mortality of 10 organisms at 0.1 ppm in 6 hours or less.

The virtues of the potency rating concept are more comprehensible through example. Table 3 lists results of a preliminary fish bioassay and defines the individual potencies at each concentration and time interval. The value at 1.0 ppm is found by using the part of formula 2 pertaining to 1.0 ppm and 3 hours exposure time, or the value may be obtained from the appendix.

$$Pr = M \times \frac{1}{1.0} \times \frac{1}{\log T} = 5 \times \frac{1}{0.4771} = 10.48$$

The sum of the individual values, 284.46, is divided by log N (1.1761) according to formula 3, and the Pr of this chemical to fish is 241.87. The Pr of 241.87 is then compared with Pmax of 491.23 in table 2, and since this 3-digit whole number is a large proportion of Pmax but not 300, mortality was assumed to have occurred at 0.1 ppm in exposures less than 24 hours.

If the chemical produced no mortality at 0.1 ppm, Pr becomes the sum of potencies at 1.0 and 10 ppm divided by log N, or 45.07/1.1761, which equals 38.32. This 2-digit whole-number Pr indicates mortality at 1.0 ppm in the shorter exposures but not complete mortality in 3 hours, since the maximum in this case is 57.25/1.1761 or 48.68. The 2-digit whole-number value also indicates no mortality at 0.1 ppm.

TABLE 3.--Potency rating determined from mortality among 10 fish

Exposure	Mortality at concentrations (ppm) of--			Contributions to Pr at concentrations (ppm) of--			Sum
	0.1	1.0	10.0	0.1	1.0	10.0	
3 hours....	0	5	8	0.00	10.48	1.68	12.16
6 hours....	5	9	10	64.25	11.57	1.29	77.11
24 hours....	9	10	10	65.21	7.25	0.72	73.18
48 hours....	10	10	10	59.48	5.95	0.59	66.02
96 hours....	10	10	10	50.45	5.04	0.50	55.99

Since the concentrations are in multiples of 10, the contribution to Pr can be calculated for one concentration, such as 0.1 ppm, for total mortality among the 10 fish. These figures can then be used for the contributions to Pr at 1.0 and 10 ppm by multiplying them by 0.1 and 0.01 and rounding off to two decimals. Once the values for mortalities are established, the procedure becomes simple and efficient.

DISCUSSION AND CONCLUSIONS

The potency rating concept is more applicable to data derived from toxicants similar in nature or ones with similar modes of action. The concept is therefore limited to materials producing dose-effect curves similar in slope. Chemicals which produce rapid effects cannot be compared accurately with ones which require longer time for results.

The potency rating concept is especially applicable to data such as those reported by Walker, Starkey, and Marking (1966) and Marking and Willford (1970). The compounds in these investigations are related to structure and activity against fish and produce similar slopes on dose-effect curves.

The formula and concept are general and can be used to include different concentrations and time exposures depending on the program. In each case, the maximum potency must be calculated and the Pr values compared with it. If larger or smaller figures are sought, the values can be multiplied by a constant to yield the desired number of digits.

Pmax is uniform at all exposure times (table 2), but Pr changes to some extent with exposure time. Pr increases with exposure time when the mortality is delayed, but the increase is much less than the additive effects of the potencies at the different exposures. If mortality occurs at 3 hours, the Pr values approximate more closely the uniformity of Pmax found in table 2.

Equal numbers of test animals must be used in trials in order to compare Pr values with a single maximum potency. Data containing

different numbers of test animals must be grouped and compared with a maximum potency for each group.

SUMMARY

Potency ratings effectively estimate the toxicity of chemicals to organisms from preliminary data which otherwise are often considered inadequate. The concept is especially useful in comparing results of numerous toxins tested against a wide variety of organisms. The values derived indicate the general and differential toxicity to the species tested.

The formula is versatile in that different concentrations and exposures can be selected to better fit a specific testing program. Mortality is a function of concentration and time, and the potency rating does not change in relation to the maximum potency if different concentrations or time intervals are selected. Data from 24- or 48-hour tests can be compared with those from 96-hour tests since the maximum potency is approximately equal for the three exposure times.

Shorter exposures and lower concentrations are emphasized in the formula by reciprocating the concentrations and the log of the time exposures. The effect is greater for highly toxic materials which kill organisms rapidly.

The potency rating concept is more applicable to data obtained from chemicals producing similar dose-effect curves. Chemicals of similar structure and chemicals which

produce similar response to organisms can be readily evaluated and compared.

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APPENDIX

Pr contributions at 0.1, 1.0, and 10.0 ppm for 10 test organisms

Exposure and concentration	Pr contributions calculated from death of--									
	1	2	3	4	5	6	7	8	9	10
3 hours:										
0.1 ppm.....	20.96	42.92	62.88	83.84	104.80	125.76	146.72	167.68	188.64	209.60
1.0 ppm.....	2.10	4.29	6.29	8.38	10.48	12.58	14.67	16.77	18.86	20.96
10.0 ppm.....	0.21	0.43	0.63	0.84	1.05	1.26	1.47	1.68	1.89	2.10
6 hours:										
0.1 ppm.....	12.85	25.70	38.55	51.40	64.25	77.10	89.95	102.80	115.65	128.50
1.0 ppm.....	1.29	2.57	3.86	5.14	6.43	7.71	9.00	10.28	11.57	12.85
10.0 ppm.....	0.13	0.26	0.39	0.51	0.64	0.77	0.90	1.03	1.16	1.29
24 hours:										
0.1 ppm.....	7.25	14.49	21.74	28.98	36.23	43.47	50.72	57.96	65.21	72.45
1.0 ppm.....	0.73	1.45	2.17	2.90	3.62	4.35	5.07	5.80	6.52	7.25
10.0 ppm.....	0.07	0.15	0.22	0.29	0.36	0.44	0.51	0.58	0.65	0.73
48 hours:										
0.1 ppm.....	5.95	11.90	17.84	23.79	29.74	35.69	41.64	47.58	53.53	59.48
1.0 ppm.....	0.60	1.19	1.78	2.38	2.97	3.57	4.16	4.76	5.35	5.95
10.0 ppm.....	0.06	0.12	0.18	0.24	0.30	0.36	0.42	0.48	0.54	0.60
96 hours:										
0.1 ppm.....	5.05	10.09	15.14	20.18	25.23	30.27	35.32	40.36	45.43	50.45
1.0 ppm.....	0.51	1.01	1.51	2.02	2.52	3.03	3.53	4.04	4.54	5.05
10.0 ppm.....	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.51

INVESTIGATIONS IN FISH CONTROL

**37. Comparative Toxicity of
29 Nitrosalicylanilides
and Related Compounds
to Eight Species of Fish**

By Leif L. Marking and Wayne A. Willford



UNITED STATES DEPARTMENT OF THE INTERIOR, WALTER J. HICKEL, *SECRETARY*
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COMPARATIVE TOXICITY OF 29 NITROSALICYLANILIDES AND RELATED COMPOUNDS TO EIGHT SPECIES OF FISH

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ABSTRACT.--The relative potencies of 29 nitrosalicylanilides and related structures against rainbow trout, goldfish, carp, fathead minnows, black bullheads, green sunfish, bluegills, and yellow perch were determined in 96-hour static bioassays. They varied from zero to the maximum potency of the system, depending on the type and position of substitutions. The 4'-substitutions on 3-nitrosalicylanilide are essential in producing high toxicity and selectivity to fish. The toxic activity of nitrosalicylanilides increases as halogen substitutions are shifted from the 2' to the 3' and to the 4' positions on the aniline moiety. Activity increases as molecular weight of the substituent increases. 4'-azophenyl-3-nitrosalicylanilide and 4'-iodo-3-nitrosalicylanilide are more toxic than 4'-bromo- or 4'-chloro-3-nitrosalicylanilide. The dihalo-substituted 3-nitrosalicylanilides are more toxic than monohalo-substituted 3-nitrosalicylanilides provided one of the halo-substitutions is at the 4'-position on the aniline moiety. 4'-bromo-3-nitrosalicylanilide is more toxic to carp, fathead minnows, bluegills, and yellow perch than to rainbow trout. Several compounds are selective to yellow perch. Goldfish are the most resistant species to the salicylanilides.

The nitrosalicylanilides are among the more biologically active chemicals and are under intensive investigation by scientists in many fields. Gönner (1962) and Schräufstatter (1962) defined the molluscicidal activity of salicylanilides and reported reduction of *Schistosomiasis* through control of the vector snail. Vinson, Dineen, and Schneider (1961) point out that salicylanilides are efficacious as hospital detergent sanitizers. Molnar and Baron (1964) stated that certain polybrominated salicylanilides possess the desirable properties of germicides to the maximum extent. Molnar (1965) reported synergistic activity against bacteria when two polybrominated salicylanilides were combined.

The salicylanilide structure is also of interest in sea lamprey control. Bayluscide, chemically known as 2',5-dichloro-4'-nitrosalicylanilide and better known as Bayer 73, is presently used to synergize 3-trifluoromethyl-4-nitrophenol (TFM) (Howell et al., 1964). Bayer 73 is highly toxic to fish, and Marking and Hogan (1967) reported 96-hour LC50's of 0.043 to 0.230 ppm. They found that the toxicity of the compound is reduced in waters of extremely high or low pH, but it is not reduced or enhanced greatly by water temperatures between 7° and 22° C.

Starkey and Howell (1965) investigated the activity of a number of salicylanilides and

related compounds against rainbow trout and larval sea lampreys and reported greater toxicity to larval lampreys in many instances.

The biological activity of salicylanilides may be enhanced or diminished by specific substitution on the molecule. Taborsky, Darker, and Kaye (1959) and Taborsky and Starkey (1962, 1963) demonstrated various antimicrobial activities of salicylanilides with selected halogens at certain positions.

Walker, Starkey, and Marking (1966) showed that the piscicidal activity of salicylanilides and related compounds is governed by the nature and location of substitutions on the molecule. The halo- substitutions on the nitro-salicylanilides, in particular, had greater influence on toxicities to fish than other substituents.

Twenty-nine nitrosalicylanilides and related structures, many having halo- substitutions at selected positions, were obtained for preliminary bioassays against eight species of fish. These tests, conducted at the Fish Control Laboratories at La Crosse, Wis. and Warm Springs, Ga., in facilities described by Lennon and Walker (1964), provide comparative data on the toxicity of the chemicals to selected freshwater fish.

MATERIALS AND METHODS

The nitrosalicylanilides and related structures were furnished by Ben Venue Laboratories, Bedford, Ohio. Each structure was chosen according to its toxicity determined previously on rainbow trout and goldfish. The general structure of the compounds is illustrated in figure 1.

The fish were obtained from National Fish Hatcheries (table 1). They were introduced to the bioassay following a routine procedure of 10-day quarantine, removal from feed, and acclimation to water chemistry and temperature as outlined by Lennon and Walker (1964).

The tests were conducted in 15 liters of standard bioassay medium at 12° C. at La Crosse and at 17° C. at Warm Springs.

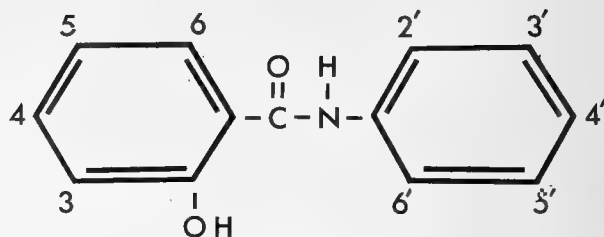


Figure 1.--Basic structure of salicylanilides. Benz-anilides lack the OH⁻ at the 2 position.

TABLE 1.--Sizes and sources of test fishes

Species and lot	Average length (inches)	Average weight (grams)	Source
Rainbow trout (<i>Salmo gairdneri</i>):			
Lot 17.....	2.0	1.2	Manchester NFH, Iowa
Lot 180.....	1.2	0.2	La Crosse FCL, Wis.
Lot 192.....	1.9	1.1	Manchester NFH, Iowa
Lot 287.....	1.9	1.1	Do.
Goldfish (<i>Carassius auratus</i>):			
Lot 19.....	--	2.4	Lake Mills NFH, Wis.
Lot W34.....	1.6	1.1	Marion NFH, Ala.
Lot 245.....	1.7	2.3	Lake Mills NFH, Wis.
Carp (<i>Cyprinus carpio</i>):			
Lot W36.....	1.3	0.7	Marion NFH, Ala.
Lot W76.....	1.5	0.6	Do.
Lot 148a.....	1.7	1.0	Lake Mills NFH, Wis.
Lot 289.....	1.6	0.8	Do.
Fathead minnow (<i>Pimephales promelas</i>):			
Lot 257.....	1.6	0.7	Do.
Lot 296.....	1.8	1.0	Do.
Black bullhead (<i>Ictalurus melas</i>):			
Lot 153.....	2.0	1.6	Necedah NWR, Wis.
Lot 237.....	1.3	0.5	Genoa NFH, Wis.
Lot 275.....	2.1	2.0	Guttenberg NFH, Iowa
Lot 290.....	2.2	2.2	Do.
Green sunfish (<i>Lepomis cyanellus</i>):			
Lot 145.....	1.4	0.8	Lake Mills NFH, Wis.
Lot 190.....	1.7	1.7	Do.
Lot 251.....	1.5	1.1	Do.
Lot 272.....	1.5	0.8	Do.
Bluegill (<i>Lepomis macrochirus</i>):			
Lot W34.....	1.5	0.9	Marion NFH, Ala.
Lot 266.....	1.2	0.4	Lake Mills NFH, Wis.
Lot 288.....	1.5	0.9	Do.
Yellow perch (<i>Perca flavescens</i>):			
Lot 151.....	2.2	1.6	Do.
Lot 267.....	2.3	1.9	Do.

Ten specimens were exposed to each concentration of 0.1, 1.0, and 10.0 ppm of the candidate compounds. A stock solution of each compound, using acetone as the solvent, was prepared immediately before each test. Test concentrations of 0.1, 1.0, and 10.0 mg/l were obtained by adding an appropriate aliquot of a stock to 15 liters of reconstituted water. Observations on the effects of the compounds were recorded at 15 and 30 minutes and at 1, 3, 24, 48, 72, and 96 hours.

The data were analyzed according to the method of Marking (1970) in which the potency rating (Pr) is defined for each chemical to

each species of fish. The values derived indicate the toxicity as a proportion of the predetermined maximum potency with respect to concentrations and observation periods. Since the observations were taken at 3, 24, 48 and 96 hours in these experiments, the Pr is found as follows:

$$Pr = \sum_{j=3,24}^{48,96} \sum_{i=0.1,}^{1.0,10.0} \frac{M(C_i, T_j)}{C_i \log T_j} \frac{1}{\log N}$$

where M = mortality among 10 fish.

C = concentration in ppm.

T = time of exposure in hours, $T > 1$.

N = number of observations, $N > 1$.

j = finite variables of T.

i = finite variables of C.

When the individual concentrations of 0.1, 1.0, and 10.0 ppm at each observation period are considered, the formula becomes

$$Pr = \frac{(M \times \frac{1}{0.1} \times \frac{1}{\log 3}) + (M \times \frac{1}{1.0} \times \frac{1}{\log 3})}{\log N} + \frac{(M \times \frac{1}{10} \times \frac{1}{\log 3}) + \dots + (M \times \frac{1}{10} \times \frac{1}{\log 96})}{\log N}$$

Pmax is obtained by assuming that all of the fish died at each concentration and observation interval, and the values are given in table 2. Pmax is 403.16 in a 96-hour bioassay, and Pr values for experimental chemicals are compared with it. The number of digits in the Pr and size of the number effectively define the potency. Three-digit whole numbers indicate mortality at 0.1 ppm. Three-digit whole numbers over 200 indicate mortality at 0.1 ppm in 3 hours, and as Pr approaches the value of Pmax a greater number of fish die at this concentration and time interval.

Two-digit whole numbers indicate mortality at 1.0 ppm, and 1-digit whole numbers

TABLE 2.--Maximum potency at four time intervals

Time	log T	$\frac{1}{\log T}$	N	log N	At concentration (ppm) of--			Sum	Pmax
					0.1	1.0	10		
3 hours	0.4771	2.0960	3	0.4771	209.60	20.96	2.10	232.66	487.65
24 hours	1.3802	0.7245	6	0.7782	72.45	7.25	0.72	80.42	402.31
48 hours	1.6812	0.5948	9	0.9542	59.48	5.95	0.59	66.02	397.30
96 hours	1.9823	0.5045	12	1.0792	50.45	5.04	0.50	55.99	403.16
Total	--	--	--	--	--	--	--	435.09	--

indicate mortality at 10 ppm. If none of the fish die at any concentration and time interval, Pr becomes zero.

RESULTS

The behavioral response of fish on contact with the nitrosalicylanilides is immediate at 10 ppm, and with some at 1.0 ppm. The response usually involves irritation, which is revealed by intermittent opercular flipping that simulates a coughing action. Swimming becomes irregular and is characterized by surfacing and diving. Loss of equilibrium and orientation are usually followed by a period of slow movement and quiescence. The opercular flipping becomes infrequent just before death.

The gill covers of dead fish are often distended, and a proliferation of mucous is noticeable about the gill area. In some cases, long strings of mucous were observed in the anal area and on the ends of the ventral fins. Microscopic examination indicates that the bodies of the dead fish are devoid of mucous. Hemorrhage of the gills occurs frequently at the higher concentrations.

The iodo- substitutions on the nitrosalicylanilide structure (table 3, compounds 1, 2, 3, and 4) are toxic to all the species tested. The degree of toxicity is progressively enhanced for most species by moving the iodo- from the 2' to the 3', and from the 3' to the 4' positions. Also, the rate of toxic action is progressive. Survival is almost complete at 1.0 ppm in 3 hours with the 2'-iodo- substitution, whereas complete mortality occurs at this concentration and exposure with the 4'-iodo- substitution. By shifting the nitro-group from the 3 position, as in compounds 1 to 3, to the 5 position (compound 4) the general activity decreases.

TABLE 3.--The potency rating of 29 chemicals to fish in bioassays at 12° C.

[Rating calculated from data in the appendix]

Chemical	Rainbow trout	Goldfish	Carp	Fathead minnow	Black bullhead	Green sunfish	Bluegill	Yellow perch
(1) 2'-iodo-3-nitrosalicylanilide...	39.95	1.68	22.47	20.52	20.52	6.79	7.01	20.52
(2) 3'-iodo-3-nitrosalicylanilide...	39.37	19.61	4.09	26.35	39.95	20.52	20.52	73.75
(3) 4'-iodo-3-nitrosalicylanilide...	241.08	39.95	208.94	208.94	208.94	112.82	202.23	247.79
(4) 4'-iodo-5-nitrosalicylanilide...	21.80	19.84	19.18	20.52	20.52	13.81	20.52	92.23
(5) 2'-bromo-3-nitrosalicylanilide..	39.95	2.26	20.52	39.95	20.52	6.33	9.65	20.52
(6) 3'-bromo-3-nitrosalicylanilide..	39.95	10.22	38.00	39.95	39.95	13.73	20.52	56.85
(7) 4'-bromo-3-nitrosalicylanilide..	39.37	20.52	403.16	128.51	87.26	39.95	99.87	228.36
(8) 4',5-dibromo-3-nitrosalicylanilide.....	208.94	38.30	67.27	186.69	158.52	83.80	169.38	208.94
(9) 4'-bromo-2'-methyl-3-nitrosalicylanilide.....	67.03	26.19	190.00	85.46	39.95	110.18	25.19	202.23
(10) 2',5-dibromo-3-nitrosalicylanilide.....	39.95	6.07	19.85	36.06	28.29	26.35	8.40	39.95
(11) 4'-chloro-3-nitrosalicylanilide.	48.18	41.53	44.30	104.90	58.65	44.62	31.82	73.75
(12) 2'-chloro-4'-methyl-3-nitrosalicylanilide.....	53.11	53.11	38.00	39.95	90.65	38.00	39.95	235.56
(13) 4'-chloro-2'-methyl-3-nitrosalicylanilide.....	39.37	19.61	133.39	45.41	39.95	22.47	20.20	175.14
(14) 2'-chloro-4'-nitro-3-nitrosalicylanilide.....	39.37	67.03	37.33	68.52	54.81	19.85	25.19	52.97
(15) 5'-chloro-2'-methoxy-3-nitrosalicylanilide.....	56.85	3.66	41.17	26.35	20.52	20.52	20.52	32.18
(16) 2',5-dimethoxy-4'-chloro-3-nitrosalicylanilide.....	39.37	52.37	74.92	26.35	20.52	17.53	29.87	95.70
(17) 2'-chloro-5'-trifluoromethyl-3-nitrosalicylanilide.....	175.14	39.95	175.14	165.20	168.66	38.00	114.49	127.92
(18) 3',4'-dichloro-3-nitrosalicylanilide.....	53.11	42.22	38.00	148.53	208.94	30.24	149.72	209.42
(19) 4'-chloro-5-bromo-3-nitrosalicylanilide.....	208.94	29.88	173.27	202.23	63.32	116.87	152.79	228.36
(20) 2'-chloro-5-nitrosalicylanilide.	39.95	2.84	11.40	16.94	17.84	18.77	3.62	11.10
(21) 3'-chloro-5-nitrosalicylanilide.	15.94	1.68	10.67	--	3.62	5.02	3.62	19.18
(22) 4'-chloro-5-nitrosalicylanilide.	21.80	20.60	15.26	20.52	32.65	20.52	20.52	46.25
(23) 2'-methoxy-4'-nitro-3-nitrosalicylanilide.....	7.01	1.68	5.85	6.60	3.43	3.35	3.08	7.06
(24) 4'-methoxy-2'-nitro-3-nitrosalicylanilide.....	20.52	18.16	4.56	15.25	20.52	4.44	3.50	20.52
(25) 2',4'-dimethyl-3-nitrosalicylanilide.....	39.95	3.62	19.85	15.39	20.52	4.56	4.56	22.47
(26) 4'-azophenyl-3-nitrosalicylanilide.....	228.36	39.37	176.58	208.94	182.09	137.50	208.94	208.94
(27) 3'-chloro-3-nitrobenzanilide....	7.47	3.62	5.30	3.62	3.23	3.62	3.43	5.30
(28) 2',6'-diethyl-3,5-dinitrobenzanilide.....	0	0	0	1.01	0	0	0	0
(29) 5-bromo-3-nitrosalicylic acid...	39.95	20.52	20.52	20.52	20.52	27.14	85.48	205.06

The 2'-, 3'-, and 4'-bromo- substitutions on the 3-nitrosalicylanilide structures (compounds 5, 6, and 7) are slightly less toxic than the equivalent iodo- substitutions with the exception of compound 7 to carp. Of the mono-bromo- substitutions, 4'-bromo-3-nitrosalicylanilide is the most toxic, killing all

species at 1.0 ppm and all carp at 0.1 ppm in 3 hours. The Pr of this chemical to carp is 403.16 and equals the Pmax of the system. Its Pr values suggest specificity for carp. All carp and yellow perch and 20 percent of the fathead minnows were killed at 0.1 ppm within 24 hours, but no mortality occurred

among the other species. At 96 hours, 90 percent of the fathead minnows and bluegills and 70 percent of the black bullheads died.

In most instances, the toxicity of the 4'-bromo- substitution is further enhanced by addition of a second bromo- on the acid portion of the molecule at the 5 position (compound 8). This congener produced 100-percent mortality in all species at 0.1 ppm within 96 hours. The rate of toxic action, however, decreases. This is evidenced by less mortality of fish at 1.0 ppm after 3 hours, and longer survival of carp at 0.1 ppm than with the mono- 4'-bromo- substitution (appendix).

Substitution of a methyl group in 2' position on 4'-bromo-3-nitrosalicylanilide does not increase the activity of the compound (compound 9). All carp and yellow perch, and 60 percent of the green sunfish died when exposed to 0.1 ppm for 96 hours (appendix). The 2',5'-dibromo- substitution (compound 10) produces approximately the same toxicity as the mono-, 2'-bromo- substitution (compound 5).

Corresponding chloro- substitutions on the 3-nitrosalicylanilide are usually less toxic than the bromo- or iodo- substitutions (compounds 11-19). This is illustrated with 4'- substitutions of the three halogens. Toxicity increases as the substitution is changed from chloro- to bromo- to iodo- (compounds 3, 7, and 11).

Methyl-, nitro-, methoxy-, or dimethoxy- substitutions on mono-chloro-3-nitrosalicylanilide fail to exhibit outstanding toxicity (compounds 11-16). However, the 2'-chloro-4'-methyl and 4'-chloro-2'-methyl structures are relatively toxic to yellow perch (compounds 12 and 13) and may have potential as selective toxicants. The Pr of cpd 12 is 235.56 and is 2 to 6 times as great as the Pr values for other species.

The 5'-trifluoromethyl substitution (compound 17) on 2'-chloro-3-nitrosalicylanilide is more toxic than mono-substituted 4'-chloro-3-nitrosalicylanilide to six of the eight species.

The 3'-chloro- or 5-bromo- substitutions (compounds 18 and 19) on 4'-chloro-3-nitrosalicylanilide also enhance the general toxicity of the molecule. The dichloro- substitution, however, failed to kill carp or green sunfish at 0.1 ppm, while the bromo- substitution killed all carp and green sunfish at 0.1 ppm in 96 hours. Both compounds killed all of the fathead minnows, bluegills, and yellow perch at 0.1 ppm in 96 hours (appendix).

The chloro-5-nitrosalicylanilides (compounds 20-22) are less toxic than the chloro-3-nitrosalicylanilides. Activity increases by relocating the chloro- from the 2' or the 3' to the 4' positions in much the same manner as demonstrated by the iodo- and bromo-3-nitrosalicylanilides.

Nonhalo- substitutions on the 3-nitrosalicylanilide produce variable results. The 2'-methoxy-4'-nitro- substitution (compound 23) appears less toxic than the 4'-methoxy-2'-nitro- and the 2',4'-dimethyl- substitutions (compounds 24 and 25). None of these are as toxic as mono- 4'-halo- substitutions.

Substitution of the larger azophenyl- radical at the 4' position (compound 26) produces greater toxicity than any of the nonhalo-, and in most cases greater than the halo- substitutions. All fish tested at 0.1 ppm died within 96 hours (appendix).

The 3'-chloro-3-nitrobenzanilide (compound 27) is far less toxic than the halo- substitutions on 3- or 5-nitrosalicylanilides. The 3'-chloro-3-nitrobenzanilide produces little mortality of any species at 1.0 ppm in 96 hours, and Pr values range from 3.23 to 7.47 for all species.

The toxicity of 2',6'-diethyl-3,5-dinitrobenzanilide (compound 28) is considerably less than that of 3'-chloro-3-nitrobenzanilide. This compound, containing multiple substitutions, fails to kill any species at 10.0 ppm in 96 hours, and consequently the Pr values are zero. One fathead minnow died unnaturally at 1.0 ppm in 48 hours, and this produces a Pr of 1.01 at 96 hours.

The 5-bromo-3-nitrosalicylic acid (compound 29), although composed of only a portion of the nitrosalicylanilide molecule, contains the vital hydroxy- in the 2 position and the nitro- in the 3 position. Its toxicity is approximately equal to or greater than that of the 2' and 3' bromo- or iodo- on 3-nitrosalicylanilides (compounds 1, 2, 5, and 6) but less than that of the 4' bromo- or iodo- (compounds 3 and 7).

DISCUSSION

The highly toxic nitrosalicylanilides are active in shorter periods than the tables indicate. In 15 to 30 minutes, 10 ppm of the 4'-halo-3-nitrosalicylanilides kill rainbow trout, fathead minnows, and yellow perch. The more potent 4'-azophenyl-3-nitrosalicylanilide kills all species tested including the relatively resistant carp, black bullheads, green sunfish, and bluegills in 30 minutes at 10 ppm. Several of these species are killed in one hour at 1.0 ppm. All of the nitrosalicylanilides at 10 ppm produce stress in trout and yellow perch within 15 minutes.

We found that the activity of the halo- substitutions increases as the atomic weight of the substituted halogen increases. This agrees with the results of Walker, Starkey, and Marking (1966). However, this order of activity does not correspond to that for fungi and bacteria. Taborsky and Starkey (1962) reported that 4'-chloro- and 4'-bromo- substitutions on the 3- and 5-nitrosalicylanilides are among the more active antimicrobials. They also observed that 4'-bromo-5-nitrosalicylanilide was a more potent antimicrobial than 4'-chloro-3-nitrosalicylanilide. Our data indicate that the 3-nitrosalicylanilides are more toxic to fish than 5-nitrosalicylanilides. The inverse antimicrobial activity of these compounds suggests their possible use in treating fish diseases of bacterial and fungal origin.

The 4'- position appears to be a key position on the molecule. The halo- substitutions at this position are toxic to all species tested. The azophenyl- substitution in this position

enhances the compound even further and suggests that bulkier unsaturated substitutions may increase the activity.

The potency ratings indicate that several chemicals are selective to target species of fish. Compound 7 (4'-bromo-3-nitrosalicylanilide) appears more toxic to carp, fathead minnows, black bullheads, bluegills, and yellow perch than to rainbow trout. Compound 12 (2'-chloro-4'-methyl-3-nitrosalicylanilide) and compound 29 (5-bromo-3-nitrosalicylic acid) are considerably more toxic to yellow perch than to any of the other species tested.

CONCLUSIONS AND SUMMARY

Nitrosalicylanilides continue to be of interest in fish control. The versatility of the parent structure permits a wide variety of substitutions enabling a critical review of the structure-activity relationships. The potency ratings on the three chemicals tested against eight species of fish indicate desirable selective toxicities. Of the 29 chemicals, 28 were toxic to fish at concentrations of 10 ppm or less.

Nitrosalicylanilides are more toxic to the eight species of fish tested than nitrobenzanilides with respective substitutions. This may be attributed to the hydroxy- at the 2 position on the benzoic acid moiety of the nitrosalicylanilides. Also, the activity of nitrosalicylanilides increases as the nitro- is shifted from the 5 to the 3 position.

The activity of a structure increases as the halogen is shifted from the 2' to the 3' and to the 4' positions on the aniline moiety. In addition, activity increases as the molecular weight of the substituent increases. Greater toxicity is observed with the heavier azophenyl- substitution, followed by iodo-, bromo-, and chloro- in that order. The activity of 4'-bromo-3-nitrosalicylanilide is enhanced further with the addition of another bromo-, providing that a bromo- remains at the 4' position.

The 5-bromo-3-nitrosalicylic acid produces toxicity which is approximately equal to

or greater than that of 2' and 3' bromo- or iodo- substitutions on the 3-nitrosalicylanilide. This molecule, although smaller and simpler, contains the hydroxy- at the 2 position which is necessary to produce maximum biological activity.

The results indicate that 4' halo- and azophenyl- substitutions on 3-nitrosalicylanilide produce the greatest toxicity against fish. These compounds deserve further consideration as fish control agents.

The relative potency concept expedited the critical analysis of these preliminary data. It permitted rapid comparison to distinguish highly toxic from less toxic chemicals and aided in distinguishing selectivity.

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APPENDIX

Toxicity of 0.1, 1.0, and 10 ppm of experimental chemicals to fish at 12° C.

[Numbers indicate mortalities among 10 fish in each bioassay]

Chemical and exposure time	Rainbow trout			Goldfish			Carp			Fathead minnow			Black bullhead			Green sunfish			Bluegill			Yellow perch		
	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
(1) 2'-iodo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	0	0	0	1	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10
24 hours.....	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	1	10	0	2	10	0	10	10
48 hours.....	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	2	10	0	10	10
96 hours.....	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	3	10	0	2	10	0	10	10
(2) 3'-iodo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	1	10	0	0	10	0	3	10	0	10	10	0	0	10	0	0	10	0	10	10
24 hours.....	0	10	10	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
48 hours.....	0	10	10	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
96 hours.....	--	--	--	--	--	--	0	1	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
(3) 4'-iodo-3-nitrosalicylanilide:																								
3 hours.....	2	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
24 hours.....	9	10	10	0	10	10	10	10	10	10	10	10	10	10	10	2	10	10	9	10	10	10	10	10
48 hours.....	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	4	10	10	10	10	10	10	10	10
96 hours.....	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	8	10	10	10	10	10	10	10	10
(4) 4'-iodo-5-nitrosalicylanilide:																								
3 hours.....	0	2	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	6	10
24 hours.....	0	10	10	0	5	10	0	8	10	0	10	10	0	10	10	0	0	10	0	10	10	3	10	10
48 hours.....	0	10	10	1	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	3	10	10
96 hours.....	--	--	--	--	--	--	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	5	10	10
(5) 2'-bromo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	0	3	0	0	10	0	10	10	0	0	10	0	0	10	0	0	10	0	0	10
24 hours.....	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	1	10	0	0	10	0	10	10
48 hours.....	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	5	10	0	10	10
96 hours.....	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	7	10	0	10	10
(6) 3'-bromo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	1	10	0	9	10	0	10	10	0	10	10	0	0	10	0	0	10	0	10	10
24 hours.....	0	10	10	0	1	10	0	10	10	0	10	10	0	10	10	0	4	10	0	10	10	1	10	10
48 hours.....	0	10	10	0	3	10	0	10	10	0	10	10	0	10	10	0	6	10	0	10	10	1	10	10
96 hours.....	0	10	10	0	5	10	0	10	10	0	10	10	0	10	10	0	9	10	0	10	10	1	10	10
(7) 4'-bromo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	0	10	10	10	10	0	10	10	0	9	10	0	10	10	0	5	10	1	10	10
24 hours.....	0	10	10	0	10	10	10	10	10	2	10	10	0	10	10	0	10	10	0	10	10	10	10	10
48 hours.....	0	10	10	0	10	10	10	10	10	6	10	10	3	10	10	0	10	10	5	10	10	10	10	10
96 hours.....	--	--	--	0	10	10	10	10	10	9	10	10	7	10	10	0	10	10	9	10	10	10	10	10
(8) 4'-bromo-5-bromo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	1	10	0	0	10	0	2	10	0	7	10	0	0	10	0	0	10	0	10	10
24 hours.....	10	10	10	0	10	10	0	10	10	9	10	10	5	10	10	0	10	10	7	10	10	10	10	10
48 hours.....	10	10	10	3	10	10	0	10	10	10	10	10	8	10	10	3	10	10	10	10	10	10	10	10
96 hours.....	10	10	10	--	--	--	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
(9) 4'-bromo-2'-methyl-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	4	10	0	10	10	0	9	10	0	10	10	0	10	10	0	0	10	0	10	10
24 hours.....	2	10	10	0	10	10	8	10	10	1	10	10	0	10	10	3	10	10	0	10	10	9	10	10
48 hours.....	2	10	10	0	10	10	9	10	10	4	10	10	0	10	10	4	10	10	0	10	10	10	10	10
96 hours.....	--	--	--	--	--	--	10	10	10	4	10	10	0	10	10	6	10	10	1	10	10	10	10	10
(10) 2',5'-dibromo-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	0	0	0	0	10	0	8	10	0	4	10	0	3	10	0	0	10	0	10	10
24 hours.....	0	10	10	0	2	10	0	9	10	0	10	10	0	10	10	0	10	10	0	2	10	0	10	10
48 hours.....	0	10	10	0	3	10	0	10	10	0	10	10	0	10	10	0	10	10	0	2	10	0	10	10
96 hours.....	0	10	10	0	3	10	0	10	10	0	10	10	0	10	10	0	10	10	0	5	10	0	10	10
(11) 4'-chloro-3-nitrosalicylanilide:																								
3 hours.....	0	9	10	0	6	10	0	7	10	0	10	10	0	10	10	0	10	10	0	1	10	0	10	10
24 hours.....	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
48 hours.....	1	10	10	0	10	10	1	10	10	5	10	10	0	10	10	0	10	10	0	10	10	2	10	10
96 hours.....	1	10	10	2	10	10	1	10	10	8	10	10	4	10	10	1	10	10	2	10	10	2	10	10
(12) 2'-chloro-4'-methyl-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	10	10	0	9	10	0	10	10	0	10	10	0	9	10	0	10	10	2	10	10
24 hours.....	1	10	10	1	10	10	0	10	10	0	10	10	3	10	10	0	10	10	0	10	10	9	10	10
48 hours.....	1	10	10	1	10	10	0	10	10	0	10	10	3	10	10	0	10	10	0	10	10	9	10	10
96 hours.....	--	--	--	--	--	--	0	10	10	0	10	10	3	10	10	0	10	10	0	10	10	10	10	10
(13) 4'-chloro-2'-methyl-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	1	10	0	8	10	0	8	10	0	10	10	0	1	10	0	0	--	0	10	10
24 hours.....	0	10	10	0	10	10	4	10	10	0	10	10	0	10	10	0	10	10	0	10	--	8	10	10
48 hours.....	0	10	10	0	10	10	6	10	10	0	10	10	0	10	10	0	10	10	0	10	--	8	10	10
96 hours.....	--	--	--	--	--	--	8	10	10	2	10	10	0	10	10	0	10	10	0	10	--	8	10	10
(14) 2'-chloro-4'-nitro-3-nitrosalicylanilide:																								
3 hours.....	0	10	10	0	0	10	0	1	10	0	7	10	0	10	10	0	0	10	0	0	10	0	8	10
24 hours.....	0	10	10	2	10	10	0	10	10	0	10	10	0	10	10	0	9	10	0	10	10	1	10	10
48 hours.....	0	10	10	2	10	10	1	10	10	2	10	10	1	10	10	0	10	10	0	10	10	1	10	10
96 hours.....	--	--	--	--	--	--	2	10	10	5	10	10	2	10	10	0	10	10	1	10	10	1	10	10

Chemical and exposure time ^a		Rainbow trout			Goldfish			Carp			Fathead minnow			Black bullhead			Green sunfish			Bluegill			Yellow perch		
		0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
(15)	5'-chloro-2'-methoxy-3-nitro-salicylanilide:																								
	3 hours.....	0	10	10	0	0	5	0	1	10	0	3	10	0	0	10	0	0	10	0	0	10	0	6	10
	24 hours.....	1	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
	48 hours.....	1	10	10	0	1	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
	96 hours.....	1	10	10	0	1	10	4	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
(16)	2',5'-dimethoxy-4'-chloro-3-nitrosalicylanilide:																								
	3 hours.....	0	10	10	1	1	10	0	6	10	0	3	10	0	0	10	0	0	5	0	0	10	0	6	10
	24 hours.....	0	10	10	1	6	10	0	6	10	0	10	10	0	10	10	0	7	10	0	10	10	2	10	10
	48 hours.....	0	10	10	1	10	10	4	10	10	0	10	10	0	10	10	0	10	10	0	10	10	4	10	10
	96 hours.....	--	--	--	--	--	--	5	10	10	0	10	10	0	10	10	0	10	10	2	10	10	6	10	10
(17)	2'-chloro-5'-trifluoromethyl-3-nitrosalicylanilide:																								
	3 hours.....	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	9	10	0	10	10	0	10	10
	24 hours.....	8	10	10	0	10	10	8	10	10	5	10	10	4	10	10	0	10	10	2	10	10	4	10	10
	48 hours.....	8	10	10	0	10	10	8	10	10	9	10	10	10	10	10	0	10	10	6	10	10	6	10	10
	96 hours.....	8	10	10	0	10	10	8	10	10	9	10	10	10	10	10	0	10	10	6	10	10	6	10	10
(18)	3',4'-dichloro-3-nitro-salicylanilide:																								
	3 hours.....	0	10	10	0	5	10	0	9	10	0	10	10	0	10	10	0	5	10	0	10	10	1	10	10
	24 hours.....	1	10	10	1	10	10	0	10	10	1	10	10	10	10	10	0	10	10	2	10	10	8	10	10
	48 hours.....	1	10	10	1	10	10	0	10	10	10	10	10	10	10	10	0	10	10	9	10	10	9	10	10
	96 hours.....	--	--	--	--	--	--	0	10	10	10	10	10	10	10	10	0	10	10	10	10	10	10	10	10
(19)	4'-chloro-5-bromo-3-nitro-salicylanilide:																								
	3 hours.....	0	10	10	0	0	10	0	2	10	0	10	10	0	10	10	0	0	10	0	2	10	1	10	10
	24 hours.....	10	10	10	0	10	10	7	10	10	9	10	10	0	1										

INVESTIGATIONS IN FISH CONTROL

**38. Toxicity of 33NCS to
Freshwater Fish and Sea Lampreys**

By Leif L. Marking, Everett L. King,
Charles R. Walker, and John H. Howell



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Fish and Wildlife Service, Charles H. Meacham, *Commissioner*
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TOXICITY OF 33NCS (3'-CHLORO-3-NITROSALICYLANILIDE) TO FRESHWATER FISH AND SEA LAMPREY

By Leif L. Marking, Everett L. King,
Charles R. Walker, and John H. Howell

ABSTRACT.--The chemical 33NCS (3'-chloro-3-nitrosalicylanilide) was evaluated as a fish control agent and as a larvicide for sea lampreys at the Fish Control Laboratories of the Bureau of Sport Fisheries and Wildlife and the Hammond Bay Biological Station of the Bureau of Commercial Fisheries. The chemical is rapidly toxic to many species. Sea lampreys, bowfin, and channel catfish are the most sensitive species. Carp are more sensitive than trouts or sunfishes. Use of 33NCS in selective control of freshwater fishes or sea lampreys requires precise control because its toxicity is strongly influenced by variations in water quality.

The salicylanilides are of great interest in fishery research because of their promise as selective controls for freshwater fish and sea lamprey larvae (Walker, Starkey, and Marking, 1966; Starkey and Howell, 1966). Among them, 33NCS (3'-chloro-3-nitrosalicylanilide) showed the greatest potential for development as a sea lamprey larvicide during early testing at the Hammond Bay Biological Station. It also showed properties of interest during preliminary screening at the Fish Control Laboratories. A cooperative investigation was initiated to evaluate 33NCS as a selective toxicant under a variety of conditions.

The importance of studying the biological activity of a compound in different environments was demonstrated repeatedly during the development of the sea lamprey larvicide TFM (3-trifluoromethyl-4-nitrophenol) and its synergist, Bayer 73 (2',5-dichloro-4'-nitrosalicylanilide) (Applegate et al., 1961;

Howell et al., 1964). Walker, Lennon, and Berger (1964) found that environmental conditions strongly influence the toxicity of antimycin. Strufe and Gönner (1962) observed that the molluscicidal activity of Bayer 73 is affected by chemical and physical factors. Later, Fox, Ritchie, and Frick (1963) determined the effect of pH on the efficacy of Bayer 73 and postulated that the inconsistencies in published lethal concentrations are the result of differences in water quality.

Recognizing that the biological activity of 33NCS may be influenced by chemical or physical factors, our bioassays were conducted in reconstituted and natural waters of different qualities. A variety of organisms including invertebrates and fish were exposed to the toxicant in laboratory or simulated field tests.

MATERIALS AND METHODS

Pharmaceutical grade 33NCS was used in all tests. Organic solvents employed in formulating the toxicant were not in excess of amounts tolerated by the test animals.

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The common and scientific names of the six kinds of invertebrates and 30 species of fish used in the bioassays are given in table 1.

Fish Control Laboratories

The facilities and procedures for routine bioassays were described by Lennon and Walker (1964).

Reconstituted deionized water was used in most of the bioassays of 33NCS, and hard and soft water was formulated by varying the

TABLE 1.--Common and scientific names of test organisms

Common name	Scientific name
Mollusks:	
Snail.....	<i>Physa</i> spp.
Freshwater clam.....	<i>Elliptio</i> spp.
Crustaceans:	
Crayfish.....	<i>Cambarus</i> spp.
Insects:	
Stonefly nymph.....	<i>Togoperia</i> spp.
Mayfly nymph.....	<i>Hexagenia</i> spp.
Dragonfly nymph.....	<i>Ophiogomphus</i> spp.
Fish:	
Sea lamprey.....	<i>Petromyzon marinus</i>
Bowfin.....	<i>Amia calva</i>
Rainbow trout.....	<i>Salmo gairdneri</i>
Brown trout.....	<i>Salmo trutta</i>
Brook trout.....	<i>Salvelinus fontinalis</i>
Lake trout.....	<i>Salvelinus namaycush</i>
Northern pike.....	<i>Esox lucius</i>
Goldfish.....	<i>Carassius auratus</i>
Carp.....	<i>Cyprinus carpio</i>
Lake chub.....	<i>Hybopsis plumbea</i>
Common shiner.....	<i>Notropis cornutus</i>
Sand shiner.....	<i>Notropis stramineus</i>
Fathead minnow.....	<i>Pimephales promelas</i>
Longnose dace.....	<i>Rhinichthys cataractae</i>
Longnose sucker.....	<i>Catostomus catostomus</i>
White sucker.....	<i>Catostomus commersoni</i>
Black bullhead.....	<i>Ictalurus melas</i>
Yellow bullhead.....	<i>Ictalurus natalis</i>
Brown bullhead.....	<i>Ictalurus nebulosus</i>
Channel catfish.....	<i>Ictalurus punctatus</i>
Trout-perch.....	<i>Percopsis omiscomaycus</i>
Rock bass.....	<i>Ambloplites rupestris</i>
Green sunfish.....	<i>Lepomis cyanellus</i>
Pumpkinseed.....	<i>Lepomis gibbosus</i>
Bluegill.....	<i>Lepomis macrochirus</i>
Smallmouth bass.....	<i>Micropterus dolomieu</i>
Largemouth bass.....	<i>Micropterus salmoides</i>
Yellow perch.....	<i>Perca flavescens</i>
Logperch.....	<i>Percina caprodes</i>
Walleye.....	<i>Stizostedion vitreum</i>

quantity of salts added (table 2). Desired pH levels were obtained with buffer systems of potassium hydrogen phthalate ($\text{KHC}_8\text{H}_4\text{O}_4$), boric acid (H_3BO_3), and sodium hydroxide (NaOH). The bioassay solutions were aerated prior to introduction of fish.

Most of the bioassays were carried out in 5-gallon glass jars containing 15 liters of water each. Bioassays with larger fish were made in plastic tanks which held up to 45 liters of water. The toxicant, 33NCS, was dissolved in acetone, and aliquots of stock solutions were pipetted directly into bioassay vessels to produce desired concentrations.

The fish in routine bioassays ranged from 1.0 to 2.5 inches in length and were obtained from National, State, and private fish hatcheries in the vicinity of La Crosse, Wis., and Warm Springs, Ga. Larger fish used in special tests were also obtained from these sources. All fish were held in quarantine for at least 10 days. Three days before testing, the fish were taken off feed and acclimated to the water chemistry and temperatures of the bioassay.

The preliminary bioassays took place in standard reconstituted water at concentrations of 0.1, 1.0, and 10 ppm of 33NCS and at 12° and 17° C. Ten fish of each species were exposed to each concentration and their responses were observed at 0.5, 1, 3, 6, 24, 48, 72, and 96 hours.

Delineative bioassays were performed at 70, 120, 170, and 220° C. in waters of various qualities. Ten fish were exposed to each of ten concentrations. The results were used to delineate concentrations which produced 50-percent mortality (LC50 values) at selected observation periods. The LC50's, confidence

TABLE 2.--Quality and composition of reconstituted water used at the Fish Control Laboratories

Classification of water	Salt added in mg/l				pH range	Total hardness ¹	Total alkalinity ¹
	NaHCO ₃	CaSO ₄	MgSO ₄	KCl			
Soft.....	12	7.5	7.5	0.75	6.4-6.8	10- 13	10- 13
Medium ²	48	30.0	30.0	3.00	7.2-7.6	40- 48	30- 35
Hard.....	192	120.0	120.0	12.00	7.6-8.0	160-180	110-120

¹As ppm CaCO₃.

²Standard reconstituted water used in routine bioassays.

intervals (C.I.), and slope functions were derived according to methods of Litchfield and Wilcoxon (1949).

Hammond Bay Biological Station

Closed system bioassays.--The water used in most tests and for holding test specimens was pumped directly from Lake Huron at Hammond Bay. The chemical and physical characteristics of the lake water fluctuate seasonally but are considered to be generally intermediate to the extremes found among tributaries of the upper Great Lakes: total alkalinity (expressed as ppm CaCO_3) is 85 to 118, pH is 7.4 to 8.3, and specific conductance is 148 to 203 micromhos at 18° C.

Special tests were made to evaluate the effect of water quality on the toxic action of 33NCS, with natural water from representative tributaries in which sea lampreys spawn.

In standard bioassays, 5 liters of water were added to 10-liter glass battery jars which were then placed in constant-temperature troughs maintained at 12.8° C. The troughs were similar to those described by Lagler (1953). The effect of temperature was determined by tests at 1.7°, 7.2°, 12.8°, 18.3°, and 23.9° C.

Two larval sea lampreys 3.5 to 5.0 inches long and two rainbow trout 3.0 to 5.0 inches long were placed in each jar and were acclimated for 1 to 2 hours before introduction of the toxicant. At least four replications were made simultaneously at each concentration.

Aliquots of a 5-percent stock solution of 33NCS in dimethyl formamide (DMF) were diluted to 1 liter and added to the test jars to establish the final 6-liter test volume and desired concentrations. All test solutions were aerated to maintain oxygen at or near saturation.

Bioassays provided data on minimum lethal (MLC100) and maximum allowable (MAC25) concentrations (Howell and Marquette, 1962). The MLC100 is the lowest concentration which produces a complete kill of larval lampreys. The highest concentration causing no greater

than a 25-percent kill among other test fish is the MAC25. The working range of 33NCS falls between these values. The ratio of working range to the MLC100 gives the permissible additional flow (PAF) and represents the amount of dilution before the MAC25 will be reduced to the MLC100. In sea lamprey control applications, a PAF less than 1 is considered to limit utility (Howell and Marquette, 1962).

Hourly observations on specimens were recorded through the first 12 hours and at the end of each assay period, usually 21 to 24 hours. The bioassays at Hammond Bay were not conducted for longer periods, since the period of exposure in sea lamprey control is seldom greater than 24 hours.

Flowing water bioassay.--Experiments with 33NCS were conducted in raceways with flowing water to simulate natural stream conditions. These also permitted the simultaneous exposure of a variety of aquatic species and larger fish than could be accommodated in the laboratory. Simulated stream tests also provided information on the physical properties of 33NCS when applied to flowing water, the type of formulations required, and the adequacy of the chemical metering devices for stream application.

Raceway tests were made in the early fall of 1964 and again in the late spring of 1965. Chemical and physical analyses of the water were made daily during the two test periods. Ranges of values for the 1964 tests were as follows: pH, 7.8 to 8.3; conductivity, 176 to 197 micromhos; total alkalinity, 97 to 106 ppm; and water temperature 5.5° to 11.1° (mean 8.2° C.). The 1965 test characteristics were: pH, 7.6 to 8.2; conductivity, 127 to 180 micromhos; total alkalinity, 96 to 108 ppm, and water temperature, between 11.1° and 19.4° C. (mean 15.6°).

The concrete raceways were 65 feet long, 6 feet wide and 30 inches deep. Water from Lake Huron was delivered to them from a surge tank which stabilized the flow. The flow was held constant at 0.34 cfs by a "V" weir. Water depth throughout the length of the raceway was maintained at 14 inches by a stop-board.

Trout were provided by State Fish Hatcheries (Michigan Department of Conservation) at Grayling and Oden, Mich., except for one lot of brown trout which was obtained in the fall of 1964 from the Rifle River, Ogemaw County, Mich. Most of the other specimens used in the raceway tests were collected from the Ocqueoc River and Bullhead and Lost lakes, Presque Isle County, Mich. Several species were from the Cheboygan River, Cheboygan County, and from a commercial bait dealer at Traverse City, Mich. All specimens were held in flowing Lake Huron water for at least 7 days before exposure to 33NCS.

Larger fish were allowed to move freely within the raceway. Smaller specimens were confined in cylindrical screen cages. Larval lampreys were placed in cages containing 3 to 5 inches of clean beach sand at least 24 hours before each test to allow sufficient time for them to establish burrows. The confinement of larval lampreys and the other smaller specimens in cages facilitated rapid and accurate observations of mortality and prevented predation by larger fish.

Stock solutions of 33NCS were prepared using DMF as the solvent. An electric fuel pump was used to apply solutions of various strengths depending on the concentration desired (Anderson, 1962).

RESULTS

Fish Control Laboratories

Preliminary investigations.--The results of early trials with 33NCS indicate a high degree of toxicity to the fish at 12° and 17° C. (table 3). The fish respond to the toxicant immediately after its introduction into the water. At lower concentrations they become mottled in appearance, are quiescent, and swim slowly to the surface or bottom. At higher concentrations they become disoriented, irritated, and excited. Erratic swimming and surfacing occurs within 15 minutes at less than 1 ppm. Close examination shows that the entire integument is devoid of mucous. Respiration is usually rapid and

TABLE 3.--Toxicity of 0.1, 1.0, and 10.0 ppm of 33NCS to fish at two temperatures

[expressed as the number of mortalities in a bioassay of 10 fish]

Temperature and species	Average length (in.)	Mortality at--											
		3 hours			24 hours			48 hours			96 hours		
		0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
<u>At 12° C.</u>													
Bowfin.....	2.2	0	10	10	10	10	10	10	10	10	10	10	10
Rainbow trout.....	1.2	0	10	10	0	10	10	0	10	10	0	10	10
Brown trout.....	1.9	0	10	10	0	10	10	0	10	10	0	10	10
Lake trout.....	2.1	0	9	10	0	10	10	0	10	10	0	10	10
Northern pike.....	2.1	0	10	10	0	10	10	--	--	--	--	--	--
Goldfish.....	1.8	0	0	10	0	9	10	0	9	10	0	10	10
Carp.....	1.5	0	9	10	7	10	10	7	10	10	8	10	10
Fathead minnow.....	1.6	0	2	10	0	10	10	0	10	10	0	10	10
Black bullhead.....	1.3	0	8	10	0	10	10	0	10	10	0	10	10
Channel catfish.....	1.7	0	10	10	10	10	10	10	10	10	10	10	10
Green sunfish.....	1.5	0	0	10	0	10	10	0	10	10	1	10	10
Bluegill.....	1.0	1	1	10	1	10	10	1	10	10	3	10	10
Yellow perch.....	2.0	0	10	10	8	10	10	8	10	10	9	10	10
Walleye.....	1.3	0	10	10	5	10	10	8	10	10	10	10	10
<u>At 17° C.</u>													
Goldfish.....	1.6	1	0	10	1	3	10	1	7	10	1	7	10
Carp.....	1.2	0	2	10	0	9	10	0	10	10	0	10	10
Fathead minnow.....	2.1	0	0	10	0	10	10	0	10	10	0	10	10
Green sunfish.....	1.9	0	3	10	0	9	10	0	10	10	0	10	10
Smallmouth bass.....	2.0	0	9	10	0	10	10	0	10	10	0	10	10

irregular, and the operculums are distended and retracted abruptly in a coughlike action in all species. The respiratory rate slows preceding death, accompanied by spastic gulps, twitching, and tetany. The response to touch and sound progressively decreases and the fish soon lose equilibrium. Death is accompanied also by the occurrence of mucous about the gills and anus. The fish have open mouths and extended operculums at the time of death.

33NCS is rapidly as well as highly toxic to fish. Ten ppm kills all species in 3 hours. One ppm kills all bowfin, rainbow trout, brown trout, northern pike, channel catfish, yellow perch, and walleye within 3 hours, and it produces partial mortality among lake trout, carp, fathead minnow, black bullhead, green sunfish, bluegill, and smallmouth bass within the same time (table 3). Goldfish, carp, and green sunfish are the only species among which mortality was not complete at 1.0 ppm in 24 hours. Even 0.1 ppm was highly toxic, and bowfin, channel catfish, and walleye died within 96 hours.

Delineative investigations.--The LC50's of 33NCS for 11 species of fish range from 0.068 to 0.830 ppm in 96 hours at 12° C.

(table 4). Channel catfish are by far the most sensitive species. Carp, yellow perch, brown trout, lake trout, rainbow trout, fathead minnow, and black bullhead are intermediate in sensitivity to 33NCS. Goldfish, green sunfish, and bluegill are more resistant.

33NCS is unique because it kills most species in a matter of minutes. Statistical expressions of LC50 are possible in 1 to 6 hours at concentrations only slightly higher than those required to kill all fish in 24 hours. Also, concentrations necessary to produce mortality are within a very narrow range at each time interval. Minute increments in concentrations delineate all-or-none effects as denoted by extremely low slope functions. This is illustrated by a very low mean slope function which ranges from 1.13 to 1.67 (table 4). In contrast, Marking (1966) found that the slope function for goldfish exposed to p,p'-DDT was 6.02.

The trout respond considerably faster to 33NCS than warmwater species, and LC50 values were established in a 1-hour exposure (fig. 1). Higher concentrations are required to kill warmwater fish in short-term exposures. For instance, it requires less than twice as much 33NCS to kill rainbow trout in 3 hours

TABLE 4.--Toxicity of 33NCS for selected fishes at 12° C.

Species	Average length (inches)	At 24 hours		At 48 hours		At 96 hours		Mean slope function
		LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	
Rainbow trout.....	1.9	0.233	0.216-0.252	0.203	0.190-0.217	0.203	0.190-0.217	1.13
Do.....	1.9	0.300	0.275-0.327	0.260	0.245-0.276	0.240	0.214-0.269	1.15
Brown trout.....	3.3	0.173	0.142-0.210	0.167	-- --	0.167	-- --	1.25
Lake trout.....	2.1	0.325	0.304-0.348	0.207	0.190-0.226	0.200	0.176-0.228	1.19
Goldfish.....	1.8	1.400	1.240-1.580	1.030	0.840-1.270	0.830	0.690-1.000	1.47
Carp.....	1.5	0.155	0.119-0.202	0.130	0.104-0.162	0.123	0.098-0.154	1.67
Fathead minnow....	1.6	0.385	0.324-0.458	0.250	0.172-0.362	0.245	0.194-0.309	1.40
Black bullhead.....	2.4	0.440	0.350-0.550	0.255	0.203-0.307	0.255	0.203-0.307	1.39
Do.....	1.3	0.380	0.339-0.426	0.328	0.285-0.377	0.260	-- --	1.19
Channel catfish....	1.7	0.085	0.075-0.096	0.068	0.050-0.071	0.068	0.050-0.071	1.27
Green sunfish.....	1.5	0.707	0.637-0.785	0.600	0.484-0.737	0.490	0.422-0.568	1.28
Do.....	1.7	0.840	0.750-0.900	0.500	0.465-0.537	0.320	0.270-0.380	1.16
Bluegill.....	1.3	0.625	0.521-0.750	0.530	0.465-0.604	0.428	0.382-0.479	1.42
Yellow perch.....	2.2	0.195	0.156-0.244	0.172	0.146-0.203	0.155	0.135-0.178	1.39

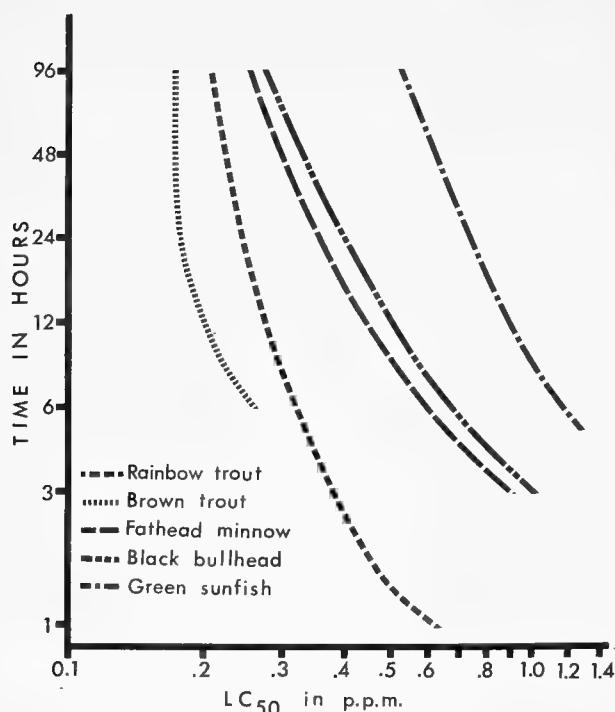


Figure 1.--Rate of response for several species of fish to 33NCS.

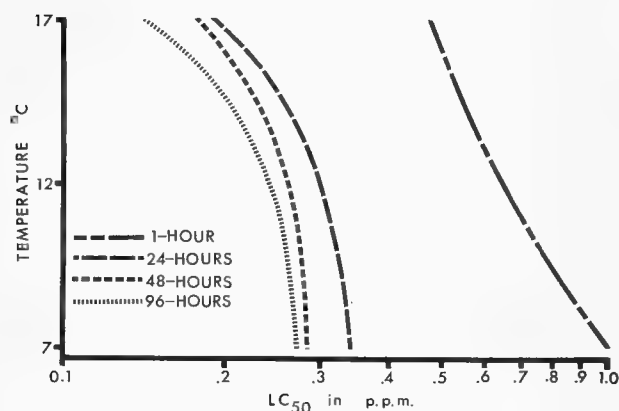


Figure 2.--Effects of temperature on the toxicity of 33NCS to rainbow trout.

as in 96 hours whereas it takes three times as much or more for carp, fathead minnow, black bullhead, and channel catfish.

Effects of temperature.--The 1-hour LC₅₀ for rainbow trout at 7° is 2.5 times that at 17° C. (fig. 2 and table 5); at 24 and 96 hours, the LC₅₀'s at 7° are less than twice those at 17°. Although the same trend prevails with green sunfish, the difference in LC₅₀ values

for 12°, 17°, and 22° is not as great. There is, however, a very similar decline in the toxic concentrations calculated at 1 to 5 hours as opposed to the 24- and 96-hour values. The data also indicate that 33NCS is more effective at warmer temperatures, and much less material is required to produce lethal effects. Examination of the slope functions at all temperatures reveals consistent values, and the narrow range of concentrations necessary to permit survival and produce mortality (all-or-none effect) prevails at all temperatures.

Effects of water quality.--The toxicity of 33NCS to rainbow trout is much greater in soft water than in hard water (table 6). The LC₅₀'s in hard water are approximately 5 times as great as those for soft water at 6, 24, 48, and 96 hours. The LC₅₀'s in water of medium hardness are about twice those for soft water. The range of pH is 6.4-6.8 for soft water and 7.6-8.0 for the hard water. Thus, pH and water hardness influence toxicity of 33NCS.

The toxicity of 33NCS to rainbow trout at all water hardnesses manifests itself within a short period of time. The LC₅₀'s at 96 hours are only slightly less than those at 6 hours. Decreases in LC₅₀ values of 28, 19, and 22 percent are noted for soft, medium, and hard water respectively.

Recognizing the variable responses in waters of different pH and hardness, we sought to identify the constituents of the reconstituted water which may affect toxicity. The toxicity of 33NCS to brook trout is considerably less when four times as much sodium bicarbonate is added to the reconstituted formula (table 7). Four times the regular amounts of potassium chloride, magnesium sulfate, or calcium sulfate had no appreciable effect on toxicity of the chemical. The addition of more sodium bicarbonate increases the pH from 7.2 to 8.2, whereas adding more of the other constituents has less effect on pH. Further, the slight increase in pH from 7.7 to 8.2 accounts for complete survival of fish at 0.5 ppm.

The specific effect of pH is demonstrated by using buffer systems at pH 5.8 and 8.5 with the standard reconstituted water (table 8). We

TABLE 5.--The toxicity of 33NCS to fish at selected temperatures

Species and exposure time	At 7° C.		At 12° C.		At 17° C.		At 22° C.	
	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.
Rainbow trout:								
1 hour.....	1.080	-- --	0.642	0.500-0.686	0.380	0.331-0.437	--	-- --
24 hours.....	0.331	0.302-0.360	0.300	0.275-0.327	0.195	0.177-0.214	--	-- --
48 hours.....	0.281	0.264-0.296	0.260	0.245-0.276	0.180	0.172-0.187	--	-- --
96 hours.....	0.275	0.262-0.288	0.240	0.214-0.269	0.142	0.123-0.163	--	-- --
Green sunfish:								
5 hours.....	--	-- --	1.220	-- --	0.870	0.820-0.920	0.715	0.660-0.775
24 hours.....	--	-- --	0.730	0.665-0.800	0.710	0.635-0.795	0.600	0.550-0.655
48 hours.....	--	-- --	0.610	0.565-0.660	0.500	0.437-0.634	0.445	0.364-0.542
96 hours.....	--	-- --	0.511	0.464-0.552	0.361	0.302-0.432	0.271	0.208-0.352

TABLE 6.--The toxicity of 33NCS to rainbow trout in various water qualities at 12° C.

Water hardness	At 6 hours		At 24 hours		At 48 hours		At 96 hours	
	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.
Soft.....	0.118	0.092-0.152	0.104	0.078-0.138	0.102	0.083-0.125	0.092	0.069-0.123
Medium.....	0.250	0.242-0.258	0.233	0.216-0.252	0.203	0.190-0.217	0.203	0.190-0.217
Hard.....	0.580	0.560-0.603	0.520	0.481-0.562	0.500	0.463-0.540	0.420	0.385-0.458

TABLE 7.--The toxicity of 0.5 ppm of 33NCS to 7.5-inch brook trout in selected water qualities

[Ten fish per test in 45-liter tanks with aeration]

Observation time	Percent mortality				
	pH 7.2 (standard bioassay water)	pH 7.5 (4 times KCl)	pH 7.6 (4 times MgSO ₄)	pH 7.7 (4 times CaSO ₄)	pH 8.2 (4 times NaHCO ₃)
1 hour.....	30	10	0	20	0
3 hours.....	30	20	20	30	0
6 hours.....	40	40	40	60	0
24 hours.....	40	40	40	60	0
96 hours.....	40	40	40	60	0

TABLE 8.--Mortality of 2.5-inch rainbow trout resulting from exposure to 33NCS at selected pH values

pH	Buffer applied	33NCS (ppm)	Percent mortality at given time
5.8.....	KHP - NaOH	0.33	100 at 30 minutes
5.8.....	KHP - NaOH	0.35	100 at 15 minutes
7.4.....	None	0.35	100 at 1 to 24 hours
8.5.....	NaOH - H ₃ BO ₃	0.35	None at 96 hours
8.5.....	NaOH - H ₃ BO ₃	0.37	30 at 96 hours

observed that 0.35 ppm of 33NCS killed all fish within 15 minutes at pH 5.8; it killed all fish in 24 hours at pH 7.4; but none died in 96 hours at pH 8.5. Thus, pH governs effectiveness and rate at which the chemical kills fish.

Brook trout which averaged 7.2 inches long were exposed in aerated vessels containing 45 liters of test solution. Erratic results such as mortality at lower concentrations and survival at higher concentrations suggested inconsistent test media. At 0.5 ppm all of the trout were dead in 1 hour, whereas 30 percent of the fish survived exposure to 0.6 ppm for 96 hours. At 0.7 ppm, 40 percent of the fish survived 1 hour, and 10 percent survived 3 hours. The swift and complete mortality at

0.5 ppm cannot be explained. However, in a subsequent bioassay using 2.5-inch rainbow trout in 15 liters of aerated test solution, the pH changed slightly owing to aeration rates. Here, 0.34 and 0.36 ppm produced 100-percent mortality in 3 hours, while 0.4 ppm produced no mortality in 48 hours and only 10 percent mortality in 96 hours. A measure of pH revealed that the values for the lower concentrations were 7.6 and 7.5 but that of the bigger concentration was 8.0. Thus, a small increment in pH of only 0.4 or 0.5 unit was responsible for the decreased activity of 33NCS.

The effects of pH and alkalinity were differentiated in bioassays conducted with 2.5-inch rainbow trout. The LC₅₀ for 96 hours using standard reconstituted water was 0.24 ppm (C.I.=0.21-0.27). An increase of pH from 7.2 to 8.2 through the use of buffers increases the LC₅₀

value to 0.35 ppm (C.I.=0.32-0.37). The toxicant in hard water, pH 8.2, had an LC50 value of 0.44 ppm (C.I.=0.41-0.47), whereas in hard water buffered to pH 7.2 the value was 0.32 ppm (C.I.=0.29-0.35) in 96 hours. These results indicate that pH apparently has slightly greater effect on the toxicity of 33NCS than does hardness.

Larger fish appear slightly more resistant to 33NCS than smaller fish. Although LC50 values are not available, 8-inch brown trout and 7.2-inch brook trout survived 0.4 ppm for 96 hours in an aerated bioassay. Smaller trout tested under regular bioassay conditions are more sensitive, and LC50's range from 0.20 to 0.24 ppm in 96 hours. Aeration increases pH, and this factor may contribute to these differences.

Several of the larger fish which had completely lost equilibrium were placed in fresh water to see whether they would recover. Two brown trout exposed to 0.6 ppm for 15 minutes regained equilibrium in 45 minutes. One of the fish died, and the other apparently recovered fully only after 24 hours in fresh water. Brook trout exposed to 0.5 ppm for 1 hour died within 10 minutes after being placed in fresh water. Thus, it appears that fish do not recover after critical exposure periods.

Effects of preexposure.--The rate of toxic action is reduced by preexposure to a sublethal level of 33NCS (table 9). Rainbow trout preexposed to 0.2 ppm of 33 NCS for 15 minutes are more resistant at 3 hours than unexposed trout. The difference in resistances at 24 hours is reduced considerably and at 48 hours it is insignificant.

TABLE 9.--The response of rainbow trout to 33NCS after preexposure to sublethal concentration

	At 3 hours		At 24 hours		At 48 hours	
	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.
Unexposed.....	0.368	0.350-0.390	0.290	0.245-0.342	0.237	0.214-0.257
Preexposed ¹	0.625	(²)	0.325	0.290-0.364	0.239	0.204-0.283

¹Preexposed to 0.2 ppm for 15 minutes.

²95-percent confidence intervals not available.

Hammond Bay Biological Station

Preliminary testing.--The toxicity of 33NCS to larval lampreys varied little from April 28, 1964, to April 22, 1965 (table 10). The MLC100 values ranged from 0.7 to 0.3 ppm (average 0.4 ppm) and exceeded 0.5 ppm only three times in the 42 assays performed.

Toxicity to fingerling rainbow trout was more variable. The MAC25 ranged from 0.8 to 2.1 ppm (average 1.3 ppm). Again the

TABLE 10.--Biological activity of a 5-percent-by-weight formulation of 33NCS in Lake Huron water in 21-hour assays at 12.8° C.

Date	Lamprey larvae MLC ₁₀₀ (ppm)	Rainbow trout MAC ₂₅ (ppm)	PAF ¹	Properties of test water		
				Conductivity (μ mhos/18° C.)	Total alkalinity (ppm CaCO ₃)	pH
1964						
Apr. 28	0.3	0.9	2.0	169.0	90.0	8.0
June 8	0.4	1.4	2.5	184.0	104.0	8.1
15	0.4	1.0	1.5	163.0	99.0	8.0
July 20	0.3	1.6	4.3	171.0	94.0	8.0
21	0.4	1.4	2.5	173.0	93.0	8.1
22	0.5	1.5	2.0	174.0	93.0	8.1
23	0.4	1.7	3.3	174.0	96.0	8.0
24	0.5	2.1	3.0	160.0	86.0	8.0
27	0.3	2.0	5.7	158.0	93.0	8.1
Aug. 4	0.6	1.6	1.7	149.0	93.0	8.2
5	0.7	1.8	1.6	172.0	96.0	8.3
6	0.3	1.8	5.0	172.0	96.0	8.1
17	0.3	1.6	4.3	186.0	103.0	8.0
18	0.4	1.4	2.5	181.0	96.0	8.3
19	0.5	1.6	2.2	181.0	96.0	8.3
20	0.3	1.4	3.7	177.0	98.0	8.3
Sept. 1	0.5	1.2	1.4	175.0	97.0	8.2
Oct. 12	0.3	1.2	3.0	-	-	-
19	0.4	1.0	1.5	-	-	-
20	0.4	1.0	1.5	-	-	-
Nov. 9	0.3	1.0	2.3	192.0	105.0	8.2
10	0.3	1.0	2.3	185.0	100.0	8.2
Dec. 2	0.4	1.0	1.5	179.0	96.0	8.1
7	0.4	0.8	1.0	156.0	85.0	7.6
14	0.5	1.2	1.4	179.0	103.0	8.0
15	0.5	0.9	0.8	159.0	96.0	7.9
17	0.6	1.2	1.0	203.0	118.0	7.8
1965						
Jan. 4	0.4	0.9	1.3	180.0	106.0	7.8
8	0.4	0.8	1.0	170.0	91.0	7.7
11	0.4	1.4	2.5	172.0	94.0	7.4
12	0.4	1.2	2.0	186.0	100.0	7.8
13	0.4	1.2	2.0	173.0	94.0	8.0
14	0.4	1.4	2.5	183.0	101.0	7.8
18	0.5	1.4	1.8	201.0	110.0	7.9
Feb. 26	0.3	1.2	3.0	190.0	98.0	7.9
Mar. 5	0.5	0.9	0.8	186.0	94.0	7.8
12	0.3	1.4	3.7	179.0	94.0	7.8
19	0.4	1.0	1.5	179.0	99.0	7.8
29	0.5	1.0	1.0	177.0	94.0	7.8
Apr. 6	0.4	0.9	1.3	175.0	95.0	7.8
13	0.5	1.0	1.0	182.0	106.0	7.8
22	0.4	0.8	1.0	148.0	85.0	7.9
Average value	0.4	1.3	2.2	175.7	97.1	8.0

¹ Permissible additional flow expressed as cubic feet per second is the ratio of working range to MLC₁₀₀ (see text for explanation).

higher values occurred infrequently; only twice did the MLC25 exceed 1.8 ppm.

The PAF values were highly variable, ranging from 0.8 to 5.0 and averaging 2.2. A PAF of less than 1.0, which is considered below usefulness for stream treatment, occurred only twice.

The data from all 42 assays show that there is some seasonal variation in toxicity of 33NCS in Lake Huron water, at least for rainbow trout. The results are therefore divided into two groups, June through October (summer) and November through April (winter).

The average ML100 of 0.42 ppm for summer and 0.41 ppm for winter assays did not differ significantly ($P > 0.04$), but the average MAC25 levels for rainbow trout of 1.49 ppm for summer and 1.07 ppm for winter assays were highly significant ($P < 0.001$). Because PAF values reflect the relation between MLC100 and MAC25, they also differed significantly ($P < 0.005$) between the two seasons (averages were 2.80 in the summer and 1.68 in the winter).

Only pH showed a significant variation ($P < 0.001$) between the two seasons (fig. 3).

TFM was more selective than 33NCS in 6 out of 9 tests in Lake Huron water (table 11), but 33NCS was 5 to 10 times as potent on larval lamprey (MLC100) and 6 to 12 times as potent on rainbow trout (MAC25) as TFM. The average PAF value was 3.0 for TFM and 2.2 for 33NCS; the MAC25's of TFM and 33NCS for rainbow trout were 2.5 to 6 times the MLC100's for lamprey larvae.

Effects of temperature.—The toxicity of 33NCS, expressed as LC50, becomes greater with each increment of test temperature (table 12). But, an examination of the 95-percent confidence limits on those values indicates that the variations are slight between 7.2° and 18.3° C. The difference observed between 1.7° and 7.2° is greater but even more so between 18.3° and 23.9° C. The slope function for each species is essentially the same and did not vary with temperature. These slope functions (1.20–1.25 for rainbow

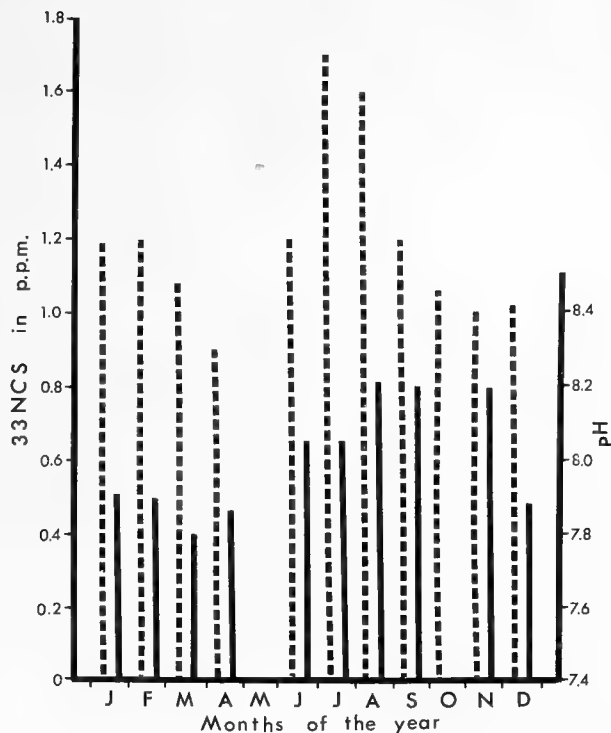


Figure 3.—Seasonal variations in pH (solid line) and concentrations of 33NCS (broken line) required to kill 25 percent (MAC25) of the rainbow trout.

TABLE 11.—Relative toxicity and selectivity of TFM and 33NCS in Lake Huron water conducted at 12.8° C. for 21 hours

Lamprey larvae			Rainbow trout			PAF	
MLC ₁₀₀	(ppm)	Potency ratio (TFM/33NCS)	MAC ₂₅	(ppm)	Potency ratio (TFM/33NCS)	TFM	33NCS
TFM	33NCS		TFM	33NCS			
3.0	0.3	10	12.0	1.8	7	3.0	5.0
3.0	0.3	10	12.0	1.4	9	3.0	3.7
2.0	0.4	5	12.0	1.0	12	5.0	1.5
3.0	0.5	6	18.0	1.6	11	5.0	2.2
3.0	0.5	6	12.0	1.2	10	3.0	1.4
3.0	0.5	6	10.0	1.4	7	2.3	1.8
4.0	0.5	8	12.0	1.0	12	2.0	1.0
4.0	0.6	7	10.0	1.6	6	1.5	1.7
4.0	0.7	6	14.0	1.8	8	2.5	1.6
Average values							
3.2	0.5	7	12.4	1.4	9	3.0	2.2

TABLE 12.—Effect of temperature on toxicity of 33NCS to larval lamprey and rainbow trout in 21-hour bioassays

Temperature (°C.)	Lamprey larvae		Rainbow trout		Slope function	
	LC ₅₀ (ppm)	95-percent C.I.	LC ₅₀ (ppm)	95-percent C.I.	Lamprey larvae	Rainbow trout
1.7°.....	0.80	0.70-0.92	1.72	1.47-2.01	1.20	1.25
7.2°.....	0.28	0.25-0.32	1.36	1.17-1.58	1.13	1.25
12.8°.....	0.26	0.22-0.30	1.20	1.07-1.34	1.25	1.22
18.3°.....	0.25	0.21-0.29	1.20	1.07-1.34	1.28	1.22
23.9°.....	0.17	0.14-0.12	0.25	0.21-0.30	1.20	1.20

TABLE 13.--Effect of temperature on toxicity, selectivity, and utility of 33NCS in 21-hour bioassays

Temperature (C.)	Lamprey larvae MLC ₁₀₀ (ppm)	Rainbow trout MAC ₂₅ (ppm)	PAF
1.7°.....	1.2	1.4	0.2
7.2°.....	0.4	1.0	1.5
12.8°.....	0.4	1.0	1.5
18.3°.....	0.4	1.0	1.5
23.9°.....	0.3	0.2	-0.3

trout and 1.13-1.28 for larval lampreys) indicate that the narrow range of concentrations required to produce an "all-or-none" response held throughout the series of temperatures used.

The MLC₁₀₀, MAC₂₅, and PAF values obtained between 7.2° and 18.3° C. are the same and indicate a usable degree of selectivity (table 13). The complete loss of selectivity observed at 23.9° C. is probably a result of unnatural stress placed on the rainbow trout rather than a true reflection of toxicity, since this temperature is near the lethal threshold for the species.

Effects of water quality.--The toxicity of 33NCS and TFM decreases with an increase in alkalinity and conductivity (table 14). The

MAC₂₅'s increase from 0.6 to 3.5 ppm for 33NCS and from 5.0 to 26.0 ppm for TFM in waters of increasing alkalinity. MLC₁₀₀ values show a parallel increase as best illustrated in figure 4. The potency ratios of 33NCS to TFM range from 4.0 to 8.5 ppm for MLC₁₀₀ and 7.1 to 10.0 ppm for MAC₂₅ in waters of various alkalinities. Therefore, alkalinity, conductivity, and pH and their interactions profoundly influence the toxicity of 33NCS and TFM to larval lamprey and rainbow trout.

Flowing water bioassay.--Of the fish used, the sea lamprey was by far the most sensitive species to 33NCS in "simulated" stream tests in 1964 and 1965 (tables 15 and 16). In contrast, the trouts are among the most resistant. The LC₅₀'s for various life stages of the sea lamprey range from 0.37 to 0.61 ppm. The calculated LC₅₀'s for other fish ranged from 0.66 to 1.75 ppm. Consistent with the laboratory tests, the narrow range of concentrations which delineate "all-or-none effects" are demonstrated by these low slope function values. Equally important is the narrow range of the confidence intervals about the LC₅₀ values.

Data on several species of fish were insufficient to calculate the LC₅₀ values, but we were able to determine the highest concentration which did not kill at least 25 percent of the test fish (table 17).

TABLE 14.--Variation in the toxicity of TFM and 33NCS in waters of various alkalinities in Michigan

[Tests conducted for 21 hours at 12.8° C]

Source of test water	Properties of test water		pH	Toxicant	Lamprey larvae MLC ₁₀₀ (ppm)	Rainbow trout MAC ₂₅ (ppm)	PAF
	Alkalinity (ppm CaCO ₃)	Conductivity (μmhos/18°C.)					
Pendills Creek, Chippewa County.....	43.0	74.0	7.6	TFM 33NCS	2.0 0.3	5.0 0.6	1.5 1.0
Lake Huron, Presque Isle County.....	97.0	175.0	8.2	TFM 33NCS	3.0 0.5	12.0 1.2	3.0 1.4
Little Billies Creek, Cheboygan County.....	148.0	225.0	8.1	TFM 33NCS	7.0 0.8	18.0 2.2	1.6 1.8
Ocequeoc River, Presque Isle County..	163.0	250.0	8.3	TFM 33NCS	5.0 0.8	16.0 2.2	2.2 1.8
Pere Marquette River, Mason County...	175.0	274.0	7.9	TFM 33NCS	5.0 1.3	18.0 2.5	2.6 1.0
Au Gres River, Iosco County.....	184.0	345.0	8.2	TFM 33NCS	7.0 1.3	25.0 3.5	2.6 1.8
Jordan River, Charlevoix County.....	189.0	304.0	8.3	TFM 33NCS	8.0 1.6	24.0 2.8	2.0 0.8
Trout River, Presque Isle County.....	200.0	310.0	8.1	TFM 33NCS	8.0 1.1	26.0 3.0	2.3 1.8

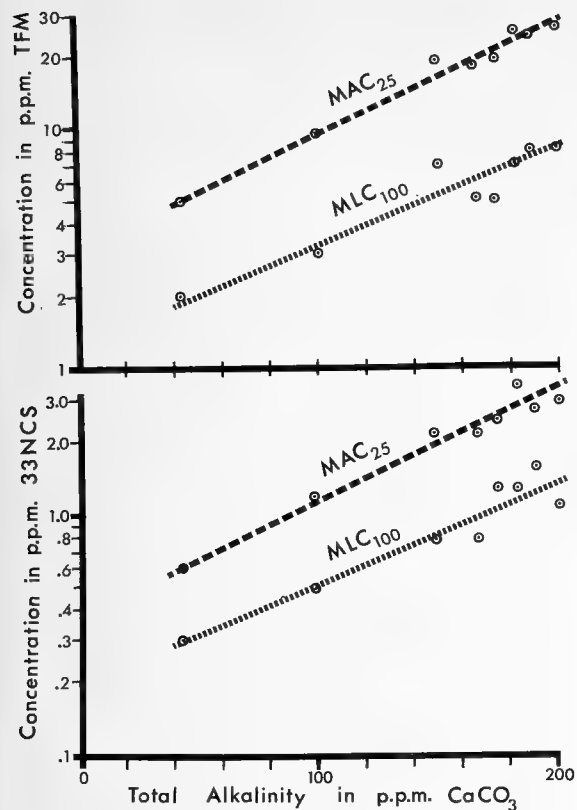


Figure 4.--The effect of alkalinity on the toxicity of 33NCS and TFM to rainbow trout and larval lamprey.

TABLE 15.--Order of toxicity of 33NCS to mature fish in 21 hours (unless otherwise specified) in simulated stream tests in fall, 1964

Test organism	LC ₅₀ (ppm)	95-percent C.I.	Slope function
Sea lamprey (transformer).	0.37	0.32-0.43	1.11
Sea lamprey (larvae).....	0.43	0.41-0.45	1.28
Sand shiner.....	0.66	0.60-0.73	1.35
Common shiner.....	0.84	0.82-0.86	1.03
Yellow perch.....	0.89	0.79-1.00	1.15
Northern pike.....	1.00	0.83-1.20	1.08
White sucker (immature)...	1.05	0.96-1.16	1.43
Fathead minnow.....	1.09	1.03-1.16	1.10
White sucker.....	1.13	0.86-1.49	1.35
Rainbow trout (immature)...	1.18	1.02-1.37	1.35
Brook trout.....	1.30	1.16-1.46	1.25
Brown trout.....	1.30	1.17-1.44	1.28
Rock bass.....	1.48	1.37-1.60	1.06
Rainbow trout.....	1.50	1.35-1.67	1.22
Brown bullhead.....	1.60	1.43-1.79	1.14
Brown trout ¹	1.65	1.29-2.11	1.48

¹ Stream-dwelling fish collected from the Rifle River, Ogemaw County, Mich.

33NCS is toxic to invertebrates, and LC50's range from 0.78 to 1.8 ppm. The LC50 value for 33NCS on stonefly nymphs was 0.78 ppm (0.64 to 0.95) in 1964 with a slope function of 1.25. The LC50 for burrowing mayfly nymphs in 1965 was 1.26 ppm (1.07 to 1.49) with a slope function of 1.34. Estimates of the

TABLE 16.--Order of toxicity of 33NCS to mature fish in 21 hours (unless otherwise specified) in simulated stream tests in spring, 1965

Test organism	LC ₅₀ (ppm)	95-percent C.I.	Slope function
Sea lamprey (larvae).....	0.38	0.37-0.40	1.22
Sea lamprey.....	0.61	0.55-0.68	1.08
White sucker.....	0.85	-	1.29
White sucker (immature)....	0.88	0.80-0.97	1.27
Yellow perch.....	0.90	0.80-1.02	1.22
Brown bullhead.....	1.14	0.94-1.38	1.20
Longnose sucker.....	1.19	1.02-1.39	1.35
Longnose dace.....	1.20	1.09-1.32	1.35
Yellow bullhead.....	1.24	1.08-1.43	1.08
Northern pike.....	1.25	0.96-1.60	1.08
Brook trout.....	1.30	1.15-1.47	1.25
Rainbow trout.....	1.31	1.16-1.48	1.15
Brown trout.....	1.50	1.32-1.71	1.23
Common shiner.....	1.75	1.59-1.94	1.20

TABLE 17.--Highest concentrations of 33NCS in ppm which did not kill more than 25 percent of the fish in simulated stream tests

Species	1964	1965
Rainbow trout (immature).....	--	1.59
Lake chub.....	--	0.40
Rock bass.....	--	1.59
Pumpkinseed sunfish (immature)...	1.80	1.59
Pumpkinseed sunfish.....	1.80	1.59
Bluegill (immature).....	1.80	1.59
Bluegill.....	1.80	1.59
Smallmouth bass.....	1.60	1.59
Largemouth bass (immature).....	1.80	1.59
Largemouth bass.....	1.80	1.00
Logperch.....	--	0.50
Walleye.....	1.20	--

LC50 values in 1964 and 1965 tests for other organisms were 1.4 and 1.26 ppm for snails, 1.4 and 1.59 for clams, and 1.8 and 1.59 for crayfish. We also estimated the LC50 to be 1.0 ppm for stoneflies and 1.59 for dragonfly nymphs in 1965.

Although the LC50 values of 33NCS varied among some species of fish from one raceway test to the other, a general pattern is evident. 33NCS is almost uniformly nontoxic to centrarchids at the levels tested. In descending order of resistance, after the Centrarchidae, are the Salmonidae, Ictaluridae, Catostomidae, Cyprinidae, Percidae, Percopsidae, and Petromyzonidae (tables 18 and 19). This listing of relative resistance to 33NCS is similar to one given by Howell (1966) for TFM.

Among the many species tested, both vertebrates and invertebrates, none is as sensitive to the toxic effect of 33NCS as the larval lamprey (LC50 = 0.38 to 0.43 ppm). The compound is also highly toxic to the lamprey at

TABLE 18.--Order of selectivity of 33NCS calculated from simulated stream tests in 1964 based on curves plotted using the data from table 15

Species	MAC ₂₅ (ppm)	PAF ¹
Sand shiner.....	0.54	-0.10
Common shiner.....	0.70	0.17
Yellow perch.....	0.82	0.37
White sucker (immature).....	0.85	0.42
White sucker.....	0.92	0.53
Northern pike.....	0.96	0.60
Rainbow trout (immature).....	0.98	0.63
Fathead minnow.....	1.02	0.70
Brown trout.....	1.09	0.82
Brook trout.....	1.14	0.90
Brown trout (stream dwelling).....	1.25	1.08
Rainbow trout.....	1.30	1.17
Rock bass.....	1.40	1.33
Brown bullhead.....	1.47	1.45

¹ Based on a MCL₁₀₀ of 0.60 ppm.

TABLE 19.--Order of selectivity of 33NCS calculated from simulated stream tests in 1965 based on curves plotted using the data from table 16

Species	MAC ₂₅ (ppm)	PAF ¹
White sucker.....	0.72	0.14
White sucker (immature).....	0.76	0.21
Yellow perch.....	0.79	0.25
Longnose sucker.....	0.96	0.52
Longnose dace.....	0.98	0.56
Brown bullhead.....	1.00	0.59
Brook trout.....	1.12	0.78
Yellow bullhead.....	1.16	0.84
Northern pike.....	1.20	0.90
Rainbow trout.....	1.20	0.90
Brown trout.....	1.30	1.06
Common shiner.....	1.55	1.46

¹ Based on a MLC₁₀₀ of 0.63 ppm.

other stages in the life cycle (LC₅₀ = 0.37 to 0.61 ppm). The trout-perch may be as susceptible as the adult lamprey to 33NCS, but the data on trout-perch are limited, and we merely state that the LC₅₀ is less than 0.50 ppm.

The LC₅₀ varied somewhat among the trouts in the two tests. The LC₅₀ for immature (3- to 5-inch) rainbow trout was 1.18 in 1964 and greater than 1.59 in 1965. The reason for this difference is unknown, but both values are within the limits observed during laboratory studies. In the 1964 race-way series, mature brown trout (7- to 14-inch) from two sources were compared. The LC₅₀ was 1.30 for hatchery stocks, whereas it was 1.65 for fish collected from a stream. The

two values, noted during the same test, illustrate that toxicity may vary among fish from different sources. In both tests, brook trout were more sensitive to 33NCS than brown or rainbow trout.

The brown bullhead was one of the most resistant species during the 1964 test (LC₅₀ = 1.60). In the second series, brown and yellow bullhead were slightly less tolerant than the trouts. The LC₅₀ values for these two species are in close agreement (1.14 for brown bullhead and 1.24 for yellow bullhead during the 1965 tests).

There were some differences in the toxicity of 33NCS to suckers and minnows in the two series of tests. These differences were small except for the common shiner. The LC₅₀ value in 1964 was 0.84 ppm, whereas the value in 1965 was 1.75. It is difficult to account for these differences on the basis of water quality, temperature, or source of test animals, and it seems more likely to be a reflection of some basic physiological difference between the specimens used in each test.

DISCUSSION

At Hammond Bay, the 21-hour LC₅₀ of 33NCS to rainbow trout in Lake Huron water was 1.2 ppm. The 24-hour LC₅₀ in standard reconstituted water at the Fish Control Laboratories was 0.3 ppm, four times the toxicity at approximately the same temperature and exposure time. The contrast is even greater in softer waters.

Bioassays on rainbow trout in harder water at La Crosse yielded an LC₅₀ of 0.52 ppm in 24 hours. Water of similar alkalinities from Pendills Creek and Lake Huron produced LC₅₀ values of 0.74 and 1.42 ppm, respectively, in aerated bioassays in 21 hours.

The apparent differences in results of bioassays at Hammond Bay and the Fish Control Laboratories are attributable to variations in the quality of aerated and non-aerated waters. Data in table 9 indicate that an increase in the bicarbonate of bioassay water, with corresponding increase in pH and

alkalinity, reduces the toxicity of 33NCS considerably. Seasonal variations in pH and alkalinity in Lake Huron water show a similar effect (table 10). For a specific concentration of toxicant, the pH alone can effect either 100 percent mortality or survival (table 8).

Aeration is a major influence on the toxicity of 33NCS; trout survive 0.5 ppm of 33NCS in reconstituted water only when the bioassay is aerated (table 7). This observation is substantiated further by the decreased toxicity in aerated bioassays at Hammond Bay.

Another factor influencing the toxicity of 33NCS is the volume of fish per volume of test solution. Heavier loads of fish in test vessels are possible at Hammond Bay because of aeration. At the heavier loadings, the ratio of toxicant per unit of biomass is considerably less than with lighter loadings. The size of test fish also is a factor. In general, larger fish are more tolerant to 33NCS.

The toxicity of 33NCS at temperatures between 7° and 18° C. showed little variation at Hammond Bay and at the Fish Control Laboratories. Toxicity to larval lamprey, rainbow trout, and green sunfish tends to increase at the higher temperatures. The extreme temperatures of 1.7° and 23.9° C. produce more drastic variations in toxicity, and such variations are more noticeable in rainbow trout than in lamprey larvae.

PAF values for 33NCS were usually less than those calculated for TFM on rainbow trout in laboratory and simulated stream trials. Lower PAF values for other species in simulated stream trials were in the descending order of resistance to 33NCS: Centrarchidae, Salmonidae, Ictaluridae, Catostomidae, Cyprinidae, and Percopsidae. These PAF values suggest that 33NCS may be limited as a selective larvicide to situations involving only trouts and sunfishes.

CONCLUSIONS

1. 33NCS is rapidly toxic to a wide variety of fishes and invertebrates.
2. The toxicity and selectivity of 33NCS are comparable under different test condi-

tions even though absolute values may vary. Sea lamprey, bowfin, carp, and channel catfish are more sensitive than trouts or sunfishes.

3. 33NCS was more toxic to fish in bioassays at the Fish Control Laboratories than at Hammond Bay. Water quality, aeration, loading, and size and source of test fish contributed to the differences in results in flowing and static bioassays.
4. The toxicity of 33NCS decreases with increasing pH, alkalinity, and hardness of water. A change in pH from 7.8 to 8.2 is very critical, and it may cause 100-percent mortality or permit survival of fish exposed to a given concentration.
5. The toxicity of 33NCS increases with temperature. At extreme temperatures the effect is more profound.
6. The regressions for 33NCS are nearly parallel for all species tested, even under different test conditions. Minute increments in concentrations delineate all-or-none effects as denoted by extremely low slope functions.
7. The toxicity of 33NCS varies with seasons. The bioassays indicate greater toxicity to rainbow trout from November through April.
8. Preexposure does not affect the ultimate lethal level but does appear to retard the rate of acute toxic action.
9. Trout did not recover after critical exposures to 33NCS.
10. 33NCS is approximately 6 times as toxic to larval lamprey as TFM in most waters tested, but 33NCS does not yield as high PAF values as TFM under the same conditions.
11. PAF values are greater than 1.0 for trouts and sunfishes in simulated stream trials. However, MLC100 values for sea lamprey and MAC25 values for other species of fish in these studies produce PAF values which may seriously limit

use of 33NCS for selective control of freshwater fish and sea lamprey larvae.

12. In field use, 33NCS would require rigid control since its performance is highly dependent on water quality.
13. The results of this study form a base for further investigation of the selective activities of 33NCS and closely related nitrosalicylanilides

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INVESTIGATIONS IN FISH CONTROL

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**39. Effects of Antimycin A on
Tissue Respiration of
Rainbow Trout and Channel Catfish**

**40. A Resume on Field Applications
of Antimycin A to Control Fish**



**United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife**

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EFFECTS OF ANTIMYCIN A ON TISSUE RESPIRATION OF RAINBOW TROUT AND CHANNEL CATFISH

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ABSTRACT.--Effects of antimycin A on respiration of liver, kidney, brain, and gill of rainbow trout and channel catfish were measured in vivo and in vitro. In vitro, brain was most resistant to the toxicant, followed by liver and kidney. Brain was more sensitive in vivo than in vitro; liver and kidney were less sensitive in vivo than in vitro. Untreated trout tissues had higher rates of respiration than did catfish tissues. Liver had the greatest oxygen consumption of all tissues.

Early investigations on the mode of action of antimycin A in animals showed it to be an inhibitor of respiration. Ahmad et al. (1949) and Ahmad, Schneider, and Strong (1950) established antimycin A as a potent inhibitor of succinate oxidation in rats. Strong (1958) states that antimycin A is a powerful and selective inhibitor of electron transport in oxidative phosphorylation. Rieske and Zaugg (1962) found almost complete inhibition of Complex III, or reduced coenzyme Q-cytochrome c reductase, by antimycin A, and determined that the antimycin-sensitive site is the segment of the respiratory chain which contains cytochrome b and c. Hepatic aldehyde oxidase is the only enzyme not in the electron transport chain that has thus far been shown to be inhibited by antimycin A (Rajagopalan, Fridovich, and Handler, 1962). Derse and Strong (1963) suggest that its mode of action in fish is also inhibition of respiration. Later, Hildebrand (1965 and 1967) showed that antimycin inhibits succinate and alpha-ketoglutarate oxidation in liver mitochondria of bluegills.

Walker, Lennon, and Berger (1964) and Berger¹ found that the toxicity of antimycin A varies among fishes. The families of trouts, perch, and herrings are most susceptible. The pikes, sunfishes, suckers, and cyprinids are of intermediate susceptibility, and the freshwater catfishes, gars, and bowfins least sensitive. Bioassays have shown that channel catfish are approximately 20 times as resistant to antimycin A poisoning as rainbow trout (Walker, Lennon, and Berger, 1964). Although the biochemical mode of action is relatively well defined, the tissue or organ sites of action in fish are not established. Also, whether the sensitivities of trout and catfish tissues to antimycin A are sufficiently different in vitro as well as in vivo to account for species selectivity has not been determined. The present study attempts to determine the tissue response of a sensitive species, the rainbow trout (Salmo gairdneri),

¹Personal communication from Bernard L. Berger, Chemist, Fish Control Laboratory, U.S. Bureau of Sport Fisheries and Wildlife, La Crosse, Wis.

and a highly resistant species, the channel catfish (*Ictalurus punctatus*), to antimycin A in vitro and in vivo.

METHODS AND MATERIALS

The rainbow trout used in these investigations ranged from 10.6 to 12.2 inches in length and from 200 to 300 grams in weight and were obtained from the National Fish Hatchery, Manchester, Iowa, and from Peterson Trout Farm, Peterson, Minn. Channel catfish were trapped from the Mississippi River by the National Fish Hatcheries at Guttenberg and Fairport, Iowa, and Genoa, Wis. They ranged from 11 to 13 inches in length and from 138 to 312 grams in weight. Both species were maintained in the laboratory as described by Hunn, Schoettger, and Whealdon (1968). Before use, all fish were acclimated to laboratory conditions in reconstituted water at 12°C, according to the methods of Lennon and Walker (1964).

Antimycin A, 98-percent active ingredient, was obtained from Ayerst Laboratories, New York, N.Y. Stock solutions for in vitro studies were prepared by dissolving antimycin A in sufficient 95-percent ethanol and diluting to volume with 50-percent ethanol. The stocks for in vivo tests were prepared by dissolving antimycin A in appropriate amounts of 100-percent acetone so that aliquots added to the test media did not exceed 1 milliliter per liter.

In vitro studies

The fish were killed by severing the spinal cord behind the head. Livers, kidneys, and brains were removed, and a small sample excised for determination of dry weight. The remainder was weighed, mixed with reaction medium, and homogenized in a teflon homogenizer. The final homogenate consisted of one part tissue (wet weight) to seven parts reaction medium. The reaction medium of Anthony and Munro (1964) was modified to contain 0.25M sucrose, 0.04M magnesium chloride, 0.02M potassium chloride, and 0.004M EDTA (tetrasodium salt). This medium gave the

most consistent and highest metabolic rates, for the tissues involved, of all media tested.

Two ml of each homogenate were placed in a standard single-arm reaction vessel with 0.2 ml of 10-percent potassium hydroxide absorbed on filter paper in the center well. One to 10 microliters of antimycin stock were added to the homogenate already in the vessels, depending on the desired concentration. Earlier, we determined that these amounts of ethanol had no effect on respiration or readings. The vessels were then placed on the manometers of a Warburg apparatus and equilibrated for 15 minutes at 25°C. Air served as the gaseous medium. No more than 10 minutes elapsed between tissue dissection and the start of equilibration.

After equilibration, the manometers were closed, and measurements were made according to the methods of Umbreit, Burris, and Stauffer (1964). Respiration rates were determined as milliliters of oxygen uptake for 1 hour per milligram dry weight of tissue ($QO_2 = O_2$ uptake per hour per mg dry weight). Two reaction vessels, one with untreated homogenate and another with reaction medium, served as controls during manometric determinations. Each experiment was replicated four or nine times, and the mean and standard deviation computed by standard methods. Analysis of variance and the method of least significant differences were used to determine whether antimycin A had caused a significant reduction (0.05 level of significance) in respiration of treated tissues compared with control values.

In vivo studies

Polyethylene tanks containing 45 liters of reconstituted water at 12°C, were used for in vivo exposures of trout and catfish to antimycin A. One fish was placed in each tank and exposed to the toxicant for 4 hours. The concentrations of antimycin A tested ranged between 3 and 80 parts per billion. Controls were exposed to corresponding concentrations of acetone solvent. The behavioral responses of the fish during exposure were recorded, and after 4 hours the fish were

killed and the tissues processed as in the in vitro experiments. The respiration of gill tissue was measured only in vivo. Two gill bars with filaments were removed from exposed fish, weighed, and placed in 2 ml of reaction medium. Gill respiration was measured as oxygen uptake per hour per mg wet weight of gill.

RESULTS

Among untreated control fish, the tissues of rainbow trout generally had higher rates of respiration than those of channel catfish at 25°C. (tables 1 and 2). This might be expected since Beamish (1964) has shown that at 20°C. the respiration of brook trout is approximately double that of brown bullheads. Oxygen consumption was greatest in liver tissue, followed by brain and kidney. The low rates of oxygen uptake by gill tissue (table 3) are not comparable to those of other tissues because they are based on wet weight.

The in vitro effect of antimycin A on the rate of oxygen uptake by tissues of rainbow trout and channel catfish appears more consistent between species than between tissues of the same species. Concentrations of 5 ppb or more of antimycin A cause a statistically significant inhibition of kidney respiration in both trout and catfish, while 10 ppb and 20 ppb interfere significantly with liver respiration (table 1). The brains of both species were approximately 4 to 8 times as resistant to the toxicant as liver. A concentration of 80 ppb lowered oxygen uptake of trout brain by 54 percent and of catfish brain by 33 percent. Although the major differences are between tissues, the degree of inhibition in catfish liver and brain is somewhat less than in trout. This suggests that tissues of the former species may be more resistant to antimycin A.

Rainbow trout and channel catfish were exposed to antimycin A in water to determine whether the concentrations of toxicant inhibiting tissue respiration in vitro also inhibited the respiratory rates of these tissues in vivo. The fish were exposed to different concentrations of the toxicant for 4 hours at 12°C. During this exposure, no observable effects were detected at concentrations of 3 ppb

on trout or 10 ppb on catfish. However, at 5 ppb the trout began surfacing after 1 to 2 hours, then settled to the bottom in a state of sedation. A similar reaction occurred in catfish within 1 hour at concentrations of 20 and 40 ppb. At 4 hours the trout had lost equilibrium and were lying on their sides, but opercular movements continued. Trout exposed to 10 ppb and catfish to 80 ppb showed symptoms of antimycin poisoning within 30 minutes and were moribund at 4 hours.

The metabolic rates of liver, kidney, brain, and gill of the above fish were measured at 25°C. In three instances, liver and kidney of trout and brain of catfish, the inhibition of respiration in vivo was similar to that in vitro (tables 1 and 2). The most striking differences between in vitro and in vivo effects of antimycin A were in the brain of trout and the liver and kidney of catfish. The concentrations causing significant inhibition in trout brain were 80 ppb in vitro compared with 10 ppb in vivo, whereas the in vitro concentrations for catfish liver and kidney were four-fold those in vivo. Gill and kidney tissue have approximately the same sensitivity to antimycin A in vivo, but the gills of catfish are between 4 and 8 times as resistant as those of trout (table 3).

DISCUSSION

The sensitivities in vitro of rainbow trout and channel catfish tissues to antimycin A are not sufficiently dissimilar to account for differences in its toxic effect on these fishes in the order of magnitude reported by Walker, Lennon, and Berger (1964), but our in vivo tests confirm the relatively high resistance of channel catfish. Thus, antimycin A is either poorly absorbed or is more readily deactivated or metabolized by catfish.

Differential rates of absorption may be linked to variations in gill structure. Steen and Berg (1966) noted significant differences between the gill structures of active fishes such as brown trout (*Salmo trutta*) and European perch (*Perca fluviatilis*), and relatively inactive species like the brown bullhead (*Ameriurus nebulosus*) and eel (*Anquilla*

Table 1.--In vitro effect of antimycin A on the respiration of three tissues of rainbow trout and channel catfish at 25°C.

Species, tissue, and concentration of antimycin	Number of fish	Oxygen uptake in μ l. of O ₂ per mg dry weight per hour				
		Mean rate	Standard deviation (\pm)	Change from control	Percent change	Significant 0.05 level
Rainbow trout:						
Liver:						
Control.....	10	3.26	0.28	--	--	--
3 ppb.....	10	3.24	0.42	-0.02	0	No
5 ppb.....	10	3.26	0.24	0	0	No
10 ppb.....	10	1.32	0.10	-1.94	59	Yes
20 ppb.....	10	1.04	0.24	-2.22	68	Yes
Kidney:						
Control.....	10	2.12	0.25	--	--	--
3 ppb.....	10	2.18	0.15	+0.06	3	No
5 ppb.....	10	1.54	0.11	-0.58	27	Yes
10 ppb.....	10	0.68	0.12	-1.44	68	Yes
20 ppb.....	10	0.45	0.10	-1.67	79	Yes
Brain:						
Control.....	5	2.51	0.21	--	--	--
5 ppb.....	5	2.51	0.07	0	0	No
10 ppb.....	5	2.40	0.26	-0.11	4	No
20 ppb.....	5	2.41	0.11	-0.10	4	No
40 ppb.....	5	2.38	0.18	-0.13	5	No
80 ppb.....	5	1.16	0.34	-1.35	54	Yes
Channel catfish:						
Liver:						
Control.....	5	1.81	0.05	--	--	--
5 ppb.....	5	1.66	0.15	-0.15	8	No
10 ppb.....	5	1.83	0.24	+0.02	1	No
20 ppb.....	5	0.97	0.15	-0.84	46	Yes
Kidney:						
Control.....	5	1.44	0.32	--	--	--
5 ppb.....	5	1.04	0.15	-0.40	28	Yes
10 ppb.....	5	0.43	0.09	-1.01	70	Yes
20 ppb.....	5	0.27	0.09	-1.17	81	Yes
Brain:						
Control.....	5	1.51	0.48	--	--	--
20 ppb.....	5	1.65	0.81	+0.14	8	No
40 ppb.....	5	1.47	0.14	-0.04	3	No
80 ppb.....	5	1.01	0.21	-0.50	33	Yes

vulgaris). They found that the lamellae on gills of inactive fish were fewer in number and thicker than those of the active fish. They calculated the diffusion distance, or distance between blood and water, in bullheads as approximately three times that in trout. Further, the investigations of Steen and Krusysse (1964) indicated that a circulatory shunt system is present in the gill lamellae of marine and freshwater teleosts. Of the freshwater forms they studied, including rainbow trout and the catfish (*Silurus glanis*), the system was most highly developed in *Silurus*. Adrenaline caused blood to circulate through the lamellae, but in the presence of acetylcholine blood passed through a central compartment directly between afferent and efferent arteries. Therefore, the resistance of catfish to antimycin A may be related to a well-

developed shunt system which is enhanced by the antimetabolic action of antimycin A, and to a thicker gill epithelium.

The in vivo and in vitro effects of antimycin A are more nearly alike in rainbow trout than in channel catfish. However, the greater sensitivity of trout brain in vivo may reflect secondary physiological effects of antimycin which are related to the biochemical actions described by Rieske (1967). Hunn² found that 0.2 mg per kg body weight of antimycin A injected intraperitoneally into carp reduced urine flows, but the urine contained elevated amounts of sodium, potassium, calcium, and

²Personal communication from Dr. Joseph B. Hunn, Fishery Biologist, Fish Control Laboratory, U.S. Bureau of Sport Fisheries and Wildlife, La Crosse, Wis.

TABLE 2.--In vivo effect of antimycin A on the respiration of three tissues of rainbow trout and channel catfish at 25°C.

[Oxygen consumption was measured at 25°C., tissues collected from fish which were exposed to antimycin A at 12°C.]

Species, tissue, and concentration of antimycin	Number of fish	Oxygen uptake in μ l. of O ₂ per mg dry weight per hour				
		Mean rate	Standard deviation (\pm)	Change from control	Percent change	Significant 0.05 level
Rainbow trout:						
Liver:						
Control.....	10	4.12	0.39	--	--	--
3 ppb.....	10	3.81	0.28	-0.31	7	No
5 ppb.....	10	3.96	0.26	-0.16	4	No
10 ppb.....	10	0.76	0.32	-3.36	82	Yes
Kidney:						
Control.....	10	2.37	0.16	--	--	--
3 ppb.....	10	2.16	0.17	-0.21	9	No
5 ppb.....	10	2.02	0.47	-0.35	15	Yes
10 ppb.....	10	0.84	0.39	-1.53	64	Yes
Brain:						
Control.....	5	2.36	0.18	--	--	--
3 ppb.....	5	2.42	0.06	+0.06	2	No
5 ppb.....	5	2.42	0.25	+0.06	2	No
10 ppb.....	5	0.65	0.25	-1.71	72	Yes
Channel catfish:						
Liver:						
Control.....	5	1.62	0.32	--	--	--
10 ppb.....	5	1.53	0.44	-0.09	6	No
20 ppb.....	5	1.74	0.27	-0.12	7	No
40 ppb.....	5	1.65	0.12	-0.03	2	No
80 ppb.....	5	1.30	0.28	-0.32	20	Yes
Kidney:						
Control.....	5	1.11	0.61	--	--	--
10 ppb.....	5	1.23	0.17	+0.12	10	No
20 ppb.....	5	0.76	0.16	-0.35	31	Yes
40 ppb.....	5	0.75	0.20	-0.36	32	Yes
80 ppb.....	5	0.56	0.19	-0.55	49	Yes
Brain:						
Control.....	5	1.57	0.14	--	--	--
10 ppb.....	5	1.50	0.15	-0.07	4	No
20 ppb.....	5	1.57	0.27	0	0	No
40 ppb.....	5	1.49	0.17	-0.08	5	No
80 ppb.....	5	0.78	0.12	-0.79	50	Yes

magnesium. Schoettger and Svendsen (1968) indicate that antimycin A disrupts the acid-base balance in rainbow trout and channel catfish. A concentration of 2 ppb reduced the total carbon dioxide content of trout blood from 26 to 9 volumes percent during a 6-hour exposure. The blood pH in these fish dropped from 7.4 to 6.9, and lactate increased from an average of 43 to 184 mg percent. Similar changes occurred in channel catfish exposed to 80 ppb for 3 hours. In later unpublished studies, we observed that the effects of antimycin A on acid-base balance in catfish were less acute until just before death. This may account for the apparently greater anesthetic effect of antimycin A on trout.

The brain tissue of mammals also appears resistant to antimycin A. According to a re-

view by Rieske (1967), the toxicant is a potent in vitro inhibitor of succinate oxidation in rat heart, kidney, brain, muscle, spleen, thymus, lung, and tumor. However, inhibition is proportional to enzyme activity in the tissue. Tissues with low succinate oxidase titer, such as spleen, lung, and thymus, were strongly inhibited in vivo. Those with high titers, such as heart, brain, and muscle, were barely affected. Liver was an exception, with a high titer and strong inhibition. The in vivo resistance of rat brain to antimycin A was explained on the basis of the blood-brain barrier. Thus, the contrasting resistances of trout and catfish brain in vivo may reflect the influence of hematological changes induced in trout by relatively low concentrations of antimycin A.

TABLE 3.--In vivo effect of antimycin A on the respiration of gill tissue of rainbow trout and channel catfish at 25°C.

[Oxygen consumption was measured at 25°C., tissues collected from fish which were exposed to antimycin A at 12°C.]

Species and concentration of antimycin	Number of fish	Oxygen uptake in μ l. of O ₂ per mg wet weight per hour				
		Mean rate	Standard deviation (\pm)	Change from control	Percent change	Significant 0.05 level
Rainbow trout:						
Control.....	10	0.125	0.019	--	--	--
3 ppb.....	10	0.126	0.009	+0.001	1	No
5 ppb.....	10	0.094	0.034	-0.031	25	Yes
10 ppb.....	10	0.015	0.008	-0.110	88	Yes
Channel catfish:						
Control.....	5	0.099	0.027	--	--	--
10 ppb.....	5	0.094	0.022	-0.005	5	No
20 ppb.....	5	0.087	0.022	-0.022	12	Yes
40 ppb.....	5	0.062	0.022	-0.037	37	Yes
80 ppb.....	5	0.036	0.011	-0.063	64	Yes

Catfishes are apparently able to exclude, detoxify, or otherwise accommodate antimycin. Resistance by exclusion was discussed earlier. However, Ritter and Strong (1966) indicated that the more resistant carp (*Cyprinus carpio*) survived longer and took up more tritium-labelled antimycin A internally than the more susceptible rainbow trout. If a portion of the radioactive residue in carp was intact antimycin, it must therefore be absorbed, but possibly at different rates since the carp survived much longer. Conceivably, limited uptake coupled with detoxication contributes to catfish resistance.

Rieske et al. (1967) demonstrated that Complex III of the mitochondrial respiratory chain is irreversibly inhibited in vitro by antimycin A. Yet, in a review of antimycin A, Rieske (1967) records other investigations which show that the succinate oxidase systems of rat liver, lung, and spleen inhibited in vivo by sublethal doses recover completely within 2 to 4 hours. Serum albumin was found to bind antimycin A and was implicated as the factor responsible for reactivation of succinate oxidase. The in vivo reactivation of respiration may result from transfer of antimycin A from the inhibited site to serum albumin and its subsequent excretion or inactivation after disassociation from albumin. Liver was the only tissue capable of chemically inactivating antimycin A in vitro. He explains this apparent discrepancy in the reversibility of antimycin A inhibition on the basis of a possible low requirement of active Complex III

to support a maximal rate of succinate oxidation. So a relatively small reactivation of this complex may be sufficient to support full succinate oxidase activity.

To date, there has been no indication of a bypass of antimycin A-inhibited components in vertebrates which could contribute to the resistance of catfishes. Takemori and King (1964) report the reversal of antimycin-inhibited succinate cytochrome *c* reductase from rat heart muscle by coenzyme Q₂. Antimycin A and coenzyme Q₂ appear competitive with regard to inhibition and reactivation respectively (Rieske, 1967), and this precludes a mechanism of reactivation involving an electronic bypass. However, Cheah (1967) reported a branched electron transport system in the cestode *Moniezia expansa* which might serve as a bypass when the normal antimycin-sensitive site was inhibited. Whether such a bypass less sensitive site or alternate pathway exists in catfishes has yet to be elucidated. Comparative investigations of biochemical and physiological mechanisms and their relation to rates of uptake and detoxication are essential to an understanding of resistance in fishes and the concurrent development of fish-control agents.

SUMMARY

The effects of antimycin A poisoning on oxygen consumption in tissues of a sensitive

species, rainbow trout, and a resistant species, channel catfish, were measured by means of a Warburg respirometer. Oxygen consumption was measured from gill exposed to antimycin A in vivo, and from liver, kidney, and brain exposed both in vivo and in vitro, at various concentrations of the toxicant.

Untreated trout tissues generally had higher rates of respiration than those of catfish at 25°C. Oxygen consumption was greatest in liver, followed by brain and kidney. In vitro effects of antimycin A were more consistent between species than tissues. Brain is four to eight times as resistant to the toxicant as liver in both species.

Trout exposed in vivo to 10 ppb and catfish to 80 ppb of antimycin A showed signs of poisoning within 30 minutes and were moribund after 4 hours at 12°C. Inhibition of liver and kidney of trout and brain of catfish in vivo was similar to that in vitro. However, trout brain was eight times as sensitive in vivo, whereas catfish liver and kidney were about one-fourth as sensitive. Catfish gill tissue was over four times as resistant than trout.

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INVESTIGATIONS IN FISH CONTROL

**40. A Resume on Field Applications
of Antimycin A to Control Fish**

By Robert E. Lennon and Bernard L. Berger



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A RESUME ON FIELD APPLICATIONS OF ANTIMYCIN A TO CONTROL FISH

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ABSTRACT.--Antimycin A, a fish toxicant, has had more than 50 applications to control fish in the field. It has been used for partial reclamations, and as a general or selective toxicant. It is effective against fish in fresh and marine waters, in acid and alkaline waters, in cold and warm waters, and in flowing and static waters. The formulations contribute no color or odor to water and do not repel fish. The toxic action, respiratory inhibition, is irreversible in most fishes. Fish-killing concentrations are harmless to most aquatic invertebrates and to higher vertebrates. Highlights of the field applications are presented.

The newest tool in fish management is antimycin A, an antibiotic produced by molds of the genus Streptomyces. Scientists at the University of Wisconsin isolated it in 1945 and thereafter tested it for years against fungi which destroy crops. During experiments in 1963, the potential of antimycin A as a fish toxicant was recognized. A use patent was obtained by the Wisconsin Alumni Research Foundation.

The antibiotic was tested exhaustively by the Bureau's Fish Control Laboratories against many species of fish in waters of diverse qualities. Concurrently, private laboratories investigated its toxicity to mice, rats, rabbits, guinea pigs, dogs, lambs, quails, pigeons, chickens, pheasants, and mallard ducks.

Antimycin is a powerful fish toxicant because minute quantities inhibit the transport of electrons at a very specific site in the respiratory system. The amounts of antimycin which kill fish are harmless to mammals and birds.

Antimycin is effective against fish, eggs to adults, in fresh and salt water, in acid and alkaline water, in clear and turbid water, and in cold and warm water. The toxic action on most species is irreversible. It evokes no spectacular response from fish. They die slowly and exhibit no frenzied activity. The toxicant contributes no odor or color to the water, and it seems to be undetected by fish. It therefore does not repel fish, which is a very important advantage in reclaiming lakes and streams.

Although stable when dry, antimycin is nonpersistent and degrades rapidly in water. It is susceptible to detoxification by small quantities of potassium permanganate.

Several formulations of antimycin were employed in experiments in the laboratory and field. A liquid formulation was used in most of the laboratory trials and in streams. Three dry formulations called Fintrol-5, Fintrol-15, and Fintrol-30 were tested in the laboratory and in lakes and ponds. These novel preparations consist of antimycin coated on sand particles in such a way that the toxicant

is released into the water as the sand sinks to the bottom. Fintrol-5 gives up its antimycin evenly within the first 5 feet of depth, Fintrol-15 within 15 feet, and Fintrol-30 within 30 feet of depth.

The Governments of the United States and Canada registered antimycin A as a fish toxicant in 1966, based on the far reaching research which demonstrates that it is effective on fish but relatively harmless to water weeds, aquatic insects, frogs, salamanders, turtles, mammals, and birds. Ayerst Laboratories has obtained registration of Fintrol-5, a shallow-water formulation of antimycin on sand. Liquid and deep-water formulations will become available to fishery managers in the near future.

FIELD TRIALS

A review of more than 50 applications of antimycin in lakes and streams in 19 States and in Canada and Guatemala indicates that it is advantageously flexible as well as effective against fish in a variety of uses and situations (tables 1-4). This summary highlights only a few of its potentials; ingenuity of fish managers may disclose other uses of the antibiotic.

Two basic criteria are involved in a lethal dose of toxicant: the concentration in water, expressed here in parts per billion (ppb), and the duration of exposure of fish to the toxicant. Although frequently overlooked, exposure is critical and must be correlated with concentration to obtain a lethal dose. Axiomatically, a low concentration must be accompanied by a long exposure to achieve a lethal dose. Conversely, a high concentration plus short exposure may equal a lethal dose. These relations must be observed with greater care when applying a toxicant to flowing waters.

Antimycin in fresh and salt waters

Most experimentation with antimycin was in fresh water and against freshwater fishes. Results are presented in later sections.

TABLE 1.--List of fishes exposed to antimycin

Abbreviation	Common name	Scientific name
Am Brk Ly	American brook lamprey	<u>Lampetra lamottei</u>
Am El	American eel	<u>Anguilla rostrata</u>
Am St	American smelt	<u>Osmerus mordax</u>
An	Anchovies	<u>Engraulidae--family</u>
At Sm	Atlantic salmon	<u>Salmo salar</u>
Bc Sh	Blackchin shiner	<u>Notropis heterodon</u>
Bf	Bowfin	<u>Amia calva</u>
Bf Hy	Buffalo hybrid	
Bg SnFh	Bluegill sunfish	<u>Lepomis macrochirus</u>
Bk BlHd	Black bullhead	<u>Ictalurus melas</u>
Blk Cr	Black crappie	<u>Pomoxis nigromaculatus</u>
Brk Sb	Brook stickleback	<u>Eucalia inconstans</u>
Brk Ss	Brook silverside	<u>Labidesthes sicculus</u>
Brk Tr	Brook trout	<u>Salvelinus fontinalis</u>
Bl CtFh	Blue catfish	<u>Ictalurus furcatus</u>
Bl Sk	Bridgelip sucker	<u>Catostomus columbianus</u>
Bm Bf	Bigmouth buffalo	<u>Ictiobus cyprinellus</u>
Bn Dc	Blacknose dace	<u>Rhinichthys atratulus</u>
Bn Mw	Bluntnose minnow	<u>Pimephales notatus</u>
Bn Sh	Blacknose shiner	<u>Notropis heterolepis</u>
Br BlHd	Brown bullhead	<u>Ictalurus nebulosus</u>
Br Tr	Brown trout	<u>Salmo trutta</u>
Bt RdHs	Blacktail redhorse	<u>Moxostoma poecilurum</u>
Ch CtFh	Channel catfish	<u>Ictalurus punctatus</u>
Chm	Chiselmouth	<u>Acrocheilus alutaceus</u>
Ch Fl	Chain pickerel	<u>Esox niger</u>
Cm Jk	Chiselmouth jack	<u>Coregonus oregonus</u>
Cm Sh	Common shiner	<u>Notropis cornutus</u>
Cn MdMw	Central mudminnow	<u>Umbra limi</u>
Co Sm	Coho salmon	<u>Oncorhynchus kisutch</u>
Cp	Carp	<u>Cyprinus carpio</u>
Cr Cb	Creek chub	<u>Semotilus atromaculatus</u>
Ct Tr	Cutthroat trout	<u>Salmo clarki</u>
Dy Vn Tr	Dolly Varden	<u>Salvelinus malma</u>
Fh CtFh	Flathead catfish	<u>Pylodictus olivaris</u>
Fh Mw	Fathead minnow	<u>Pimephales promelas</u>
Fl Fh	Fallfish	<u>Semotilus corporalis</u>
Fl SnFh	Flier sunfish	<u>Centrarchus macropterus</u>
Ft Dr	Fantail darter	<u>Etheostoma flabellare</u>
Fw Dm	Freshwater drum	<u>Aplodinotus grunniens</u>
Gd Sh	Golden shiner	<u>Notemigonus crysoleucas</u>
Gf	Goldfish	<u>Carassius auratus</u>
Gr Cp	Grass carp	<u>Ctenopharyngodon idellus</u>
Gr Fl	Grass pickerel	<u>Esox americanus vermiculatus</u>
Gr SnFh	Green sunfish	<u>Lepomis cyanellus</u>
Gz Sd	Gizzard shad	<u>Dorosoma cepedianum</u>
Hy SnFh	Hybrid sunfish	
Ia Dr	Iowa darter	<u>Etheostoma exile</u>
Jh Dr	Johnny darter	<u>Etheostoma nigrum</u>
Jk	Jacks	<u>Carangidae--family</u>
Kk Sm	Kokanee salmon	<u>Oncorhynchus nerka</u>
Le SnFh	Longear sunfish	<u>Lepomis megalotis</u>
Lk Cb Sk	Lake chub sucker	<u>Erimyzon sucetta</u>
Lk Tr	Lake trout	<u>Salvelinus namaycush</u>
Lm Bs	Largemouth bass	<u>Micropterus salmoides</u>
Ln Dc	Longnose dace	<u>Rhinichthys cataractae</u>
Ln Gr	Longnose gar	<u>Lepisosteus osseus</u>
Ln KlFh	Longnose killfish	<u>Fundulus similis</u>
Ln Sh	Longnose shiner	<u>Notropis longirostris</u>
Ln Sk	Longnose sucker	<u>Catostomus catostomus</u>
Is Sk	Largescale sucker	<u>Catostomus macrocheilus</u>
Mq Fh	Mosquito fish	<u>Gambusia affinis</u>
Mt Sp	Mottled sculpin	<u>Cottus bairdi</u>
No Hg Sk	Northern hog sucker	<u>Hypentelium nigricans</u>
No Pk	Northern pike	<u>Esox lucius</u>

TABLE 1.--List of fishes exposed to antimycin--Continued

Abbreviation	Common name	Scientific name
No Rb Dc	Northern redbelly dace	<u>Chrosomus eos</u>
No RdHs	Northern redborse	<u>Moxostoma macrolepidotum</u>
No SqFh	Northern squawfish	<u>Ptychocheilus oregonensis</u>
Os Sf	Orangespotted sunfish	<u>Lepomis humilis</u>
Qb	Quillback	<u>Carpododes cyprinus</u>
Pd Fh	Paddlefish	<u>Polyodon spathula</u>
Pm	Peamouth	<u>Mylocheilus caurinus</u>
Pp	Pompano	<u>Carangidae--family</u>
Pb SnFh	Pumpkinseed	<u>Lepomis gibbosus</u>
Rb SnFh	Redbreast sunfish	<u>Lepomis auitus</u>
Rb Tr	Rainbow trout	<u>Salmo gairdneri</u>
Re SnFh	Redear sunfish	<u>Lepomis microlophus</u>
Rk Bs	Rock bass	<u>Ambloplites rupestris</u>
Rs Sh	Redside shiner	<u>Richardsonius balteatus</u>
S CtFh	Sea catfish	<u>Galeichthys felis</u>
Sm Bf	Smallmouth buffalo	<u>Ictiobus bubalus</u>
Sm Bs	Smallmouth bass	<u>Micropterus dolomieu</u>
Sh Mw	Sheepshead minnow	<u>Cyprinodon variegatus</u>
Sh TpMw	Starhead topminnow	<u>Fundulus notti</u>
Sn Gr	Shortnose gar	<u>Lepisosteus platostomus</u>
S Ly	Sea lamprey	<u>Petromyzon marinus</u>
So Rb Dc	Southern redbelly dace	<u>Chrosomus erythrogaster</u>
Sp Gr	Spotted gar	<u>Lepisosteus oculatus</u>
Sp SnFh	Spotted sunfish	<u>Lepomis punctatus</u>
Sp Sk	Spotted sucker	<u>Minytrema melanops</u>
Spt	Spot	<u>Leiostomus xanthurus</u>
St Rr	Stoneroller	<u>Campostoma anomalum</u>
Tl Sh	Taillight shiner	<u>Notropis maculatus</u>
Tp	Tilapia	<u>Tilapia mossambica</u>
Tp Mt	Tadpole madtom	<u>Noturus gyrinus</u>
Tr Sp	Torrent sculpin	<u>Cottus rhotheus</u>
Wd Sh	Weed shiner	<u>Notropis texanus</u>
We	Walleye	<u>Stizostedion vitreum vitreum</u>
Wm Bs	Warmouth bass	<u>Chaenobryttus gulosus</u>
Wt Bs	White bass	<u>Morone chrysops</u>
Wt Cr	White crappie	<u>Pomoxis annularis</u>
Wt CtFh	White catfish	<u>Ictalurus catus</u>
Wt Sk	White sucker	<u>Catostomus commersoni</u>
Yw BlHd	Yellow bullhead	<u>Ictalurus natalis</u>
Yw Ph	Yellow perch	<u>Perca flavescens</u>

The Bureau of Commercial Fisheries has exposed marine fish to antimycin at the Biological Laboratory, Gulf Breeze, Fla. and in a tidal basin near Fort Desoto, Fla. Thirty-eight species in the basin received a 6-hour--a tidal cycle--exposure to 7 ppb of antimycin. It killed the more sensitive species such as anchovies, triggerfish, flounders, and rays. The more resistant catfishes, common jack, and redbelly did not all succumb during this brief exposure.

Antimycin in acid water

Antimycin persists longer in acid water than in alkaline water, and the application rate may be reduced.

The acid-water trials included trout ponds in northern New Hampshire and Quebec and panfish ponds in northern Wisconsin and Georgia. In New Hampshire, 1 ppb of antimycin eliminated 12 species of fish from a pond within 72 hours. Those killed included Atlantic salmon, chain pickerel, minnows, white sucker, pumpkinseeds, largemouth bass, and yellow perch. A few bluegills and smallmouth bass and all brown bullheads survived. In another pond, 12 ppb quickly killed all species except bullheads within 48 hours, the antimycin persisting for 2 weeks at toxic level.

In Quebec, 5 ppb of antimycin eradicated 13 species from Beauty Lake. The principal target fish in this 44-acre brook-trout lake were northern pike, minnows, suckers, pumpkinseeds, and yellow perch.

The trials in acid waters in eastern U.S. also confirmed the results of tests in the laboratory, demonstrating that catfishes, such as bullheads, are not harmed by small concentrations of antimycin. The complete survival of catfishes was thus expected in subsequent field trials which involved less than 25 ppb of antimycin.

Antimycin in alkaline water

Most field applications of antimycin have been in alkaline waters which ranged from pH 7.0 to 9.3. They confirmed that the toxicant is pH-sensitive, with the more rapid degradation occurring at higher pH's. Whereas rapid degradation can be exploited to great advantage in many situations where there are multiple uses of the lakes or streams, the duration of exposure to an effective concentration of toxicant must be calculated carefully to secure a lethal result on the target fish. In general greater concentrations of toxicant are required at pH 8.5 or higher to offset rapid degradation.

In 35-acre Lake Katrine near Madison, Wis., 7.5 ppb of antimycin caused a complete kill of carp and fathead minnows at pH 8.2 and 56° F. In 63-acre Parker Lake, Wis.,

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TABLE 2.--Sites of field applications with data on water quality

Trial	Date	Location	Area or volume	Temperature (°F.)	pH	Hardness (ppm)	Alkalinity (ppm)
1.....	10/63	Research pond, Delafield, Wis.	0.75 a.	65	8.5	213	210
2-4.....	9/64	Hatchery ponds, NFH, Berlin, N.H.					
		Pond No. 1	0.45 a.-ft.	53	7.0	10	14
		Pond No. 2	1.16 a.-ft.	53	7.2	10	13
		Pond No. 3	0.55 a.-ft.	53	7.2	10	15
5.....	9/64	Pond, Fort Benning, Ga.	0.23 a.	83	6.9	9	12
6-8.....	9/64	Hatchery ponds, NFH, Cape Vincent, N.Y.					
		Pond No. 1	1.0 a.	53	8.1	153	110
		Pond No. 2	1.0 a.	53	7.9	159	118
		Pond No. 3	1.0 a.	53	8.1	155	118
9-11...	3/65	Research ponds, Stuttgart, Ark.					
		Pond No. 1	0.25 a.	63	8.0	59	79
		Pond No. 2	0.25 a.	63	8.1	58	74
		Pond No. 3	0.25 a.	63	8.2	55	75
12-13..	5/65	Natural ponds, NWR, Valentine, Nebr.					
		Pond No. 1	1.8 a.	65	8.4	107	155
		Pond No. 2	0.5 a.	70	7.9	142	258
14.....	8/65	Sidie Hollow Creek, Viroqua, Wis.	1.8 cfs.	53	8.3	240	--
15.....	10/65	Veteran's Memorial Pond, West Salem, Wis.	5.0 a.	56	7.8	223	188
16.....	10/65	Lake Creek Lake, NFH, Saratoga, Wyo.	2.0 a.	48	7.7	237	184
17-18..	12/65	Research ponds, Stuttgart, Ark.					
		Pond No. 1	22 a.	53	8.6	112	110
		Pond No. 2	0.25 a.	50	8.6	120	110
19.....	2/66	Lake Atitlan, Guatemala	2.5 a.	72	7.7	--	163
20.....	5/66	Barney Lake, Madison, Wis.	34 a.	68	8.5	26	--
21.....	5/66	Katrine Lake, Madison, Wis.	34 a.	63	8.4	32	25
22.....	7/66	Alto Creek, Fox Lake, Wis.	4.9 cfs.	60	7.7	hard	--
23.....	7/66	Drew Creek, Fox Lake, Wis.	7.2 cfs.	60	7.8	hard	--
24.....	8/66	Unknown Lake, Oneida County, Wis.	--	72	5.2	4	--
25.....	8/66	Perch Lake, Wis.	--	74	neutral	soft	--
26.....	8/66	Harriet Lake, Wis.	--	76	neutral	soft	--
27-29..	8/66	Farm ponds,					
		Pond No. 1	0.94 a.	82	8.5	20	22
		Pond No. 2	1.26 a.	86	8.5	20	19
		Pond No. 3	1.17 a.	86	8.5	14	20
30.....	9/66	Mullet River, Plymouth, Wis.	10.2 cfs.	70	8.3	210	--
31.....	10/66	Barney Lake, Madison, Wis.	35 a.	56	8.2	--	35
32.....	10/66	Parker Lake, Adams County, Wis.	63 a.	48	8.2	197	--
33.....	10/66	Beauty Lake, Quebec	50 a.	60	7.0	60	75
34.....	11/66	Rathbone Creek, Cataract, Wis.	10.7 cfs.	48	7.5	24	--
35.....	1967	Catfish pond, Baton Rouge, La.	1.2 a.	74	7.2	--	--
36.....	5/67	Catfish rearing ponds, Greenwood, Mo.	28 a.	64	7.5	--	--
37.....	6/67	Backbone Lake, Delaware County, Ia.	125 a.	68	8.3	--	--
38.....	6/67	Catfish rearing ponds, Leland, Miss.	10-15 a.	78	high	50	--

TABLE 2.--Sites of field applications with data on water quality--Continued

Trial	Date	Location	Area or volume	Temperature (°F.)	pH	Hardness (ppm)	Alkalinity (ppm)
39.....	6/67	Westfield Creek, Westfield, Wis.	22 cfs.	77	8.8	--	170
40.....	7/67	Research pond, Stuttgart, Ark.	0.3 cfs.	82	8.3	--	--
41.....	8/67	Pleasant Lake, Minn.	20 a.	67	8.0	--	--
42.....	8/67	Delbert Gnegy Pond, Oakland, Md.	0.43 a.	65	8.6	80	--
43.....	9/67	Golf Course Pond, Shaw Air Force Base, S.C.	3.9 a.	79	7.2	15	16
44.....	9/67	Tarrant Lake, Cambria, Wis.	0.5 a.-ft.	50	7.7	--	270
45.....	9/67	Hatchery ponds, Avoca, Ind.	0.4 a.	72	7.5	--	--
46.....	9/67	Turquoise Lake, Leadville, Colo.	1250 a.-ft.	56	8.0	53 TDS	19
47.....	9/67	Farm ponds, Warm Springs, Ga.	0.4-1.0 a.	75	7.3	15	14
48.....	10/67	Bigfoot Beach Lagoon, Lake Geneva, Wis.	7.5 a.	54	7.9	--	260
49.....	10/67	Gold-tailing ponds, Powder River Rehabilitation, Sumter, Oreg.	0.02-0.34 a.	55	7.6	soft	--
50.....	10/67	Victory Pond, Fort Benning, Ga.	26 a.	70	7.0	--	--
51.....	10/67	Barney Lake, Madison, Wis.	34 a.	57	8.7	--	35
52.....	10/67	Whitewater Lake, NWR, Valentine, Nebr.	490 a.	62	9.3	204	310

10 ppb wiped out carp, pumpkinseed, bluegill, and yellow perch at pH 8.2 and 48°. The application of 10 ppb of toxicant in 34-acre Barney Lake, Madison, Wis., killed all carp at pH 8.7 and 57°. On the other hand, 7.3 ppb of antimycin in Whitewater Lake, Valentine, Nebr., at pH 9.3 degraded before most of the carp and other fish had sufficient exposure to kill them. Bioassays indicated that 12 to 15 ppb of toxicant were needed to produce a kill at the high pH.

Antimycin in cold and warm waters

In general, the response of fish to antimycin is slower in cold water than in warm water because of reduced metabolic activity. Thus, to insure adequate duration of exposure before degradation of the compound, a higher concentration of antimycin may be required when temperatures are below 50° F.

The performance of antimycin in cold water was observed best in simulated field tests in

1/100-acre pools at the Fish Control Laboratory, La Crosse, Wis. Brown trout, carp, bluegills, and largemouth bass were killed by 5 ppb within 2 to 7 days at pH 7.4 to 8.2 and 38° to 42° F. Other tests under ice in 1,000-gallon vinyl pools demonstrated that antimycin at 5 ppb killed rainbow trout, brown trout, small carp, bluegills, and longear sunfish. Survivors included large goldfish, large carp, fathead minnows, and pumpkinseeds. Thus, a greater concentration of toxicant would have been needed to kill all fish. Other things being equal, lower concentrations of toxicant and more rapid response of fish can be expected in water over 60° F.

Nonrepellency of antimycin

The formulations of antimycin coated on sand cause no color or odor when applied to water. Fish make no attempt to avoid contact with treated water--a great advantage when reclaiming springfed lakes and barrier-free streams.

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TABLE 3.--Results with antimycin as a general toxicant

Trial site	Objective	Species (refer to table 1)	Anti-mycin (ppb)	Formulation	Results (kill)
<u>pH below 7.5--temp. 60° F. or above</u>					
5.....	Determine effect on species present	GrPl, GdSh, LnSh, TlSh, MqF, BkSb, AmEl, FlSf, RbSf, GrSf, OsSf	0.6	Technical	Total except bluegill, warmouth, and catfishes.
33.....	Eradicate northern pike and stunted sunfish	RbTr, BkTr, AmSt, NoPk, GdSh, BcSh, BnSh, WdSh, BlSk, BlCtF, WtB, SmBs, JhDr	5.0	Fintrol 5, 15, 30 & liquid	Total except bullheads.
55.....	Determine effect of low concentration on species present	GzSd, GrPl, GdSh, BtRdH, AmEl, FlSf, BgSf, ReSf, WtCr	0.9	Liquid	Total on GzSd; partial on all other species.
53.....	Determine effect of low concentration on species present	ChPl, TlSh, ShTpMw, BgSf, ReSf, SpSf, LmBs, BrSs	0.4	Liquid	Partial on all species.
<u>pH below 7.5--temp. below 60° F.</u>					
54.....	Determine effect of low concentration on species present	ChPl, LkChSk, AmEl, BgSf, ReSf, LmBs, WtCr	1.5	Liquid	Partial on all species.
2-4.....	Determine effect on species present	RbTr, AtSm, BrTr, BkTr, ChPl, GdSh, CmSh, NoSqF, RsSh, CrCb, BlSk, BlCtF, WtB, FlSf, PsSf, OsSf, SmBs	0.13	Fintrol	Total only on common shiner.
	"	"	1.22	Fintrol	Total except bluegill, largemouth bass, and brown bullhead.
	"	"	12.1	Fintrol	Total except brown bullhead.
34.....	Eradicate white sucker	AmBkLy, BkTr, CnMlMn, BlSk, ShMw, RybSf	7.5	Fintrol-5 & liquid	99 percent.
<u>pH 7.5-8.5--temp. above 60° F.</u>					
9-11...	Determine effect on species present	RbTr, Cp, GdSh, LkCbSk, NoHgSk, SmBf, BkRh, MqF, AmEl, FlSf, OsSf, LeSf	5.0	Fintrol-5	Total except catfishes and warmouth.
	"	"	7.5	Fintrol-5	Total except catfishes.
	"	"	10.0	Fintrol-5	Total except catfishes.
45.....	Determine effect on species present	SpGr, Bf, GzSd, Chm, Cp, FlF, BlSk, LkCbSk, NoRdH, WtCtF, BkRh, MqF, AmEl, WtB, FlSf, OsSf, SmBs	5.0	Fintrol-5	Total except spotted gar, channel catfish, and bullheads.
30.....	Eradicate carp from a stream	CnMn, NoPk, Cp, GdSh, RsSh, BlSk, LnKf, AmEl, FlSf, OsSf, LeSf	7.5	Fintrol-5 & liquid	99 percent.
12.....	Eradicate all fish	NoPk, Cp, BnMw, SctF, AmEl, FlSf, OsSf, BgSf, SmBs	10.0	Fintrol-5	Total except northern pike, bullhead, largemouth bass, and black crappie.
13.....	Eradicate all fish	NoPk, Cp, BnMw, SctF, FlSf, OsSf, SmBs	10.0	Fintrol-5	Total except black bullhead.
19.....	Eradicate largemouth bass from a grebe sanctuary	OsSf, LeSf	10.0	Fintrol-5	Total on largemouth bass and black crappie.
<u>pH 7.5-8.5--temp. below 60° F.</u>					
6-8.....	Determine effect on species present	Bf, RbTr, BrTr, BkTr, Chm, Cp, GdSh, BlSk, BlCtF, ShTpM, LnKf, AmEl, WtB, FlSf, PsSf, LeSf, SmBs, LgBs, IaDr	1.0	Fintrol	Total only on trouts, minnows, and white sucker.
	"	"	3.1	Fintrol	Total except bowfin, bullheads, pumpkinseed, and largemouth bass.
	"	"	10.0	Fintrol	Total except bowfin and bullheads.
46....	Eradicate white and long-nose suckers	KkSm, ClTr, RbTr, BrTr, BkTr, SoRbD, Qb, BlSk	3.5	Fintrol-5 & liquid	65 percent.
48....	Eradicate carp	Cp	5.0	Fintrol-5	Total.

TABLE 3.--Results with antimycin as a general toxicant--Continued

Trial site	Objective	Species (refer to table 1)	Anti-mycin (ppb)	Formulation	Results (kill)
<u>pH 7.5-8.5--temp. below 60°F.--Continued</u>					
49.....	Eradicate squawfish, suckers, shiners	CmJk, RbTr, BkTr, DyVnTr, Pm, NoSqF, BnD, LnD, YwPh	7.5	Fintrol-5	Total on target species.
15.....	Eradicate total except catfish	RbTr, BrTr, CnMlMn, NoPk, Cp, GdSh, BnMw, BlSk, SCtF, BkSh, AmEL, WtB, FlSf	10.0	Fintrol-5	Total except channel catfish and bullheads.
16.....	Eradicate all fish from hatchery water supply	BkTr, BnMw, RsSh, ReSf	10.0	Fintrol-5	Total.
22.....	Eradicate carp in stream	Bf, CnMlMn, NoPk, Cp, NoSqF, BnD, BlSk, ShMw, FlSf	10.0	Fintrol & liquid	Total
23.....	Eradicate carp in stream	Cp	10.0	Fintrol & liquid	99 percent.
31.....	Eradicate carp	Cp, BnMw	7.5	Fintrol-5	Total.
32.....	Eradicate all fish	RbTr, Cp, WtB, FlSf, SmBs	10.0	Fintrol 5, 10, 15	Total.
14.....	Eradicate all fish	RbTr, BrTr, StRr, NoSqF, BnD, RsSh, BlSk, LsSk, ShMw, HybSf, JhDr	15.0	Technical	Total.
44.....	Eradicate carp and black bullhead	Cp, SCtF	150.0	Fintrol & liquid	Total.
<u>pH above 8.5--temp. 60°F. or above</u>					
52.....	Eradicate carp	ChPl, LsSk, ChCtF	7.3	Fintrol-5	Small kill.
1.....	Delineate effect on species present	AmBkly, Bf, RbTr, NoPk, Gf, Cp, BlSk, SCtF, WtCtF, AmEL, WtB, FlSf, GrSf, OsSf, LeSf, SmBs, LmBs, IaDr	10.0	Technical	Total except longnose gar, bowfin, and black bullhead.
39.....	Eradicate carp and white sucker	AmBkly, RbTr, Cp, WdSh, BnMw, NoSqF, RsSh, BlSk, ShMw, WtB, FlSf, OsSf, SpSf, HybSf, JhDr	10.0	Fintrol & liquid	99 percent.
<u>pH above 8.5--temp. below 60°F.</u>					
17.....	Eradicate all fish	Pf, GzSd, Gf, Cp, NoHgSk, BtRhH, SCtF, WtCtF, BkSh, YwBh, BrBh, ChCtF, MqF, AmEL, RkB, FlSf, OsSf, LeSf	10.0	Fintrol-5	Total except for 7 species of catfish.
18.....	Eradicate all fish	Pf, Cp, GdSh, LkCbSk, NoHgSk, BkSh, MqF, AmEL, RkB, FlSf, OsSf	10.0	Fintrol-5	Total on scale fish.
51.....	Eradicate carp	Cp	10.0	Fintrol & liquid	Total.

The clear and colorless water of Lake Creek Lake at Saratoga, Wyo., NFH afforded a fine opportunity to test the nonrepellency of antimycin. The 1.8-acre lake is entirely spring fed, and its discharge of 3 cfs supplies the hatchery with water. Our objective was to rid the lake of fish which might serve as vectors for diseases. Previous attempts with chlorine and rotenone failed to eradicate the fish because they fled into bottom springs and other springs beneath undercut banks.

The spring flow affected exchange of water in the basin of the lake every 27 hours; thus maintenance of adequate exposure to a toxicant

was most critical. Therefore, we maintained at least 10 ppb of antimycin in the lake for a minimum of 8 hours. A seed spreader achieved even distribution of the sand formulation which sank readily into the bottom springs.

The fish were clearly seen to exhibit no alarm or tendency to escape from the treated water. We first observed toxic effects 2 hours after applying antimycin: Dense schools of large northern creek chubs began to break up and seemed to lose their orientation with respect to schooling. None sought relief in spring flows, and most were dead within 24 hours.

TABLE 4.--Results with antimycin as a selective toxicant

Trial site	Objective	Species (refer to table 1)	Anti-mycin (ppb)	Formulation	Results (kill)
<u>pH below 7.5--temp. 60°F. or above</u>					
24.....	Eradicate yellow perch and small bluegill	NoPk, WdSh, SctF, LnkF, FlSf, PsSf	1.0-2.0	Fintrol-5	Total on yellow perch and partial on bluegill.
25.....	Eradicate yellow perch	NoPk, GdSh, WdSh, SctF, LnkF, PsSf	0.5	Fintrol-5	Total on yellow perch.
26.....	Eradicate yellow perch	NoPk, GdSh, WdSh, SctF, LnkF, PsSf	0.5	Fintrol-5	Total on yellow perch.
47.....	Thinning sunfish in bass-bluegill pond	GdSh, BlCtF, BkSh, FhCtF, BkSb, RkB, FlSf, HbSf, OsSf, BgSf	0.4-1.0	Fintrol-5	0.4 to 0.6 ppb gave excellent control.
35.....	Eradicate scale fish from catfish pond	GdSh, BkSh, FhCtF, AmEl, OsSf	3.0	Fintrol-5	Total on scalefishes.
50.....	Eradicate scale fish from catfish pond	GdSh, BkSh, MqF, FlSf, OsSf	3.6	Fintrol-5	Total except channel catfish, bullhead, and gambusia.
<u>pH 7.5-8.5--temp. above 60°F.</u>					
21.....	Kill carp eggs in pond	Cp	4.0	Fintrol-5	Total on eggs in area.
37.....	Eradicate adult carp in spawning bays	Cp, FlSf, OsSf, LeSf	50.0	Fintrol-5	Partial control.
36.....	Eradicate scale fish from catfish pond	GdSh, AmEl, FlSf, OsSf	5.0	Fintrol-5	Total on golden shiner, bass, bluegill, and green sunfish.
43.....	Eradicate scale fish from catfish pond	BkSh, FlSf	5.0	Fintrol-5	Total on bluegills.
41.....	Eradicate minnows from trout lake	HbTr, SoHbD, GdSh, BnSh, WdSh, NoSqF, HlSk, ShMw	5.0	Fintrol-5, -15	Total on minnows.
<u>pH above 8.5--temp. 60°F. or above</u>					
20.....	Kill carp eggs	Cp	2.0	Technical	Partial kill on eggs.
38.....	Eradicate scale fish from catfish pond	GdSh, BkSh, AmEl	4.0	Fintrol-5	Total on scalefish.
27-29..	Eradicate scale fish from catfish pond	GdSh, MqF, AmEl, FlSf	5.0	Fintrol-5	99 percent scalefish.
	"	"	7.5	Fintrol-5	99 percent scalefish.
	"	"	10.0	Fintrol-5	Total on scalefish.
42.....	Eradicate stunted sunfish	BkSh, YwSh	7.5	Fintrol-5	Total.
40.....	Reduce gizzard shad in spot treatments	GzSi, AmEl	1.5-6.5 ppm	Fintrol & liquid	Reduced population.

Brook trout in the lake ranging from one-third ounce to 6.6 pounds, easily observed throughout the reclamation, began to die 3 hours after exposure. None attempted to escape from the lake via the outlet or to move into the springs. Numerous fathead minnows and the small numbers of Iowa darters responded similarly to the creek chubs and brook trout. Antimycin killed all the fish and was flushed from the basin by spring flows within 48 hours.

Westfield Creek in south-central Wisconsin also provided an opportunity to observe the

behavior of fish under exposure to antimycin. Here the principal target fish was carp, a species particularly adept at escaping exposure to toxicants which contain repelling ingredients. We treated a 3.5-mile portion of Westfield Creek which had an average flow of 17.2 cfs, pH 8.8, total alkalinity of 170 ppm, and a temperature of 77°F. The water was clear and colorless, and had an average velocity of 0.5 feet per second. Antimycin in liquid formulation was maintained at 10 ppb for 10 hours by drip stations and spray pumps throughout the 3.5 miles of stream.

None of the numerous fish present attempted to avoid contact with the toxicant by fleeing downstream. Rather, fish gradually drifted downstream hours later when the antimycin had rendered them incapable of maintaining positions in the current. Fantail and johnny darters began to drift downstream and to die within 2 hours, followed soon by fathead and bluntnose minnows and blacknose dace, and later by common shiners, creek chubs, white suckers, bluegills, pumpkinseeds, and largemouth bass. Distressed and dying carp by the thousands began to drift involuntarily downstream after 8 hours. Brook lampreys and slimy sculpins were the last species to exhibit distress and die.

Most of the carp were dead within 24 hours. The few living specimens, all in distress, perished within 48 hours. None recovered from the toxicant, although it had flushed out of the stream hours before. Intensive electrofishing on 1.5 miles of the stream a week later failed to turn up any carp, suckers, or panfish. A few small minnows, a brook stickleback, and several brook lampreys were the only living fish found. The reclamation was an unqualified success.

Antimycin in partial reclamations

Antimycin is better suited to partial reclamation of lakes than any toxicant heretofore available. Its advantages here are (1) the lack of repellency and (2) the effectiveness on all life stages of fish, eggs through adults.

Two soft-water, high-pH lakes near Madison, Wis., were used in early experiments. Treatments of shoreline spawning areas and a bay in Lake Barney with 2 ppb of antimycin failed to kill all carp eggs. But concurrently the treatment of a spawning bay in nearby Lake Katrine with 4 ppb of antimycin resulted in complete mortality of carp eggs. Eggs in control areas of the lake hatched, and fry were easily collected; only dead eggs were found in the treated bay, and no fry could be collected.

Large numbers of carp congregated on spawning sites in Backbone Lake in Iowa were exposed to 50 ppb of antimycin. We assumed that this concentration would be

lethal to both adults and spawn if they remained exposed for 8 hours or more. The staked sites ranged from a fourth of an acre to 5 acres in area, and the toxicant was applied at night while fish were moving into the spawning areas. Within hours after the application, a brief but violent storm brought 2 inches of rain and the lake rose 3 inches in level, causing a dilution of toxicant and cessation of spawning activity. The trial demonstrated, however, that carp on the spawning sites or moving into them were not repelled by the antimycin, and many succumbed.

Another approach to partial reclamation was made in a 22-acre lake at the Fish Farming Experimental Station at Stuttgart, Ark. Here we wanted to reduce large numbers of gizzard shad which at times congregated at the inlet to the pond, but without harming the channel catfish in the pond. The first treatment involved a 6-minute titration of 50 cc of liquid antimycin into the 140-gpm inlet flow. The shad dispersed after 1.5 minutes, but they had already sustained a lethal exposure and large numbers died. Two days later a 1.5-minute titration of 50 cc of toxicant in the same flow killed large numbers of shad which had migrated into the vicinity of the inlet. A third application with 100 cc of antimycin was made 2 days later to kill a new congregation of shad, and on this occasion some green sunfish perished. The treatments were effective and economical in reducing the numbers of gizzard shad without harming channel catfish.

McGrath Lake in northern Wisconsin afforded an opportunity to attempt partial control of stunted panfish. The lake had a pH of 5.2, total hardness of 4 ppm, and a temperature of 70° F. Antimycin applied to one of the well-defined bays at 1 ppb killed all of the stunted yellow perch and 50 percent of the stunted bluegills. Another bay received 2 ppb of the toxicant, and all perch and bluegills were eliminated.

Antimycin as a selective toxicant

Fish species differ in their sensitivity to antimycin, thus offering an opportunity in many situations to practice selective control of target fish. Stunted yellow perch, for

example, were the targets for selective kills in Harriet and Perch lakes in northern Wisconsin. The waters of both lakes at the time were soft, acid, and warm. As intended, antimycin at 0.5 ppb killed all of the stunted perch without harm to northern pike, bluntnose minnows, bullheads, largemouth bass, and smallmouth bass.

Worthwhile reductions of overabundant minnows, sunfish, and crappies have also been accomplished in soft-water farm ponds in Georgia with no significant harm to largemouth bass. Applications of antimycin ranging from 0.4 to 0.8 ppb were most successful in ponds of 1 to 9 acres.

A typical operation involved a 2.5-acre bass-bluegill pond in which we needed to reduce the numbers of overabundant bluegills, golden shiners, and crappies with minimum damage to the largemouth bass. Only 0.4 ppb of antimycin applied to the 75° F. water killed 127 pounds of golden shiners, 172 pounds of stunted bluegills, 22 pounds of fingerling crappies, and only 7 pounds of fingerling bass. No adult bass and crappies were killed. Moreover, subsequent reproduction by adult bass exposed to sublethal concentrations of toxicant was not impaired.

Antimycin's greatest potential as a selective toxicant possibly lies in the removal of scalefish from catfish waters. Minnows and sunfish, for example, infest many catfish production ponds in southeast and southcentral States, consuming large quantities of catfish rations, carrying diseases which affect the welfare of catfish, and necessitating costly sorting of scalefish and catfish at harvest.

Experiments at a catfish farm in Mississippi showed the benefits of reducing or eliminating abundant scalefish from production ponds. The ponds ranged from 0.9 to 1.4 acres, and averaged 120 pounds per acre of golden shiners and green sunfish. Ten ppb of antimycin killed at least 99 percent of the scalefish, despite high pH levels. No catfish perished. At harvest, treated ponds contained more and larger catfish than untreated ponds. The improved yield of catfish per acre averaged 330 pounds more in treated than in untreated ponds.

An excellent demonstration of selective poisoning was done by the Bureau's Division of Fishery Services on Victory Lake, Fort Benning, Ga. Elimination of scale fish from the 26-acre lake would permit effective management of channel catfish on a fed basis. Antimycin in liquid and sand formulations was used: 3.6 ppb in the lake proper and 10 ppb for brief periods in tributary streams and springs. This killed more than 5,200 pounds of scalefish, or 210 pounds per acre, comprising 60 pounds of chub suckers, 33 pounds of golden shiners, 55 pounds of bluegills and warmouth, and 19 pounds of largemouth bass. The small concentration had no effect on mosquitofish. Only one channel catfish and one bullhead were killed. Subsequent netting disclosed channel catfish up to 4.5 pounds and bullheads in good condition but no other species except mosquitofish. The job was a success, and intensive management of the catfish was begun soon after.

FORMULATIONS OF ANTIMYCIN

Handy formulations have been developed with antimycin without noxious or repelling carriers. The liquid formulation of antimycin is easily distributed in shallow waters and streams with spray apparatus or drip equipment. The dry formulations consist of antimycin coated on 40-mesh sand and designed for shallow ponds and littoral zones of lakes. Fintrol-5 is a preparation which releases the antimycin evenly within the first 5 feet of depth as the sand sinks to the bottom. It is handily distributed by seed spreaders mounted on boats or helicopters. Its advantage over liquid toxicant is that the granules do not stick to emergent or shoreline vegetation, but bounce off into the water. Moreover, the sand penetrates beds of submerged vegetation and better distributes the toxicant.

Fintrol-15 formulation is designed to release the toxicant into the water within the first 15 feet of depth. The selective removal of minnows from Pleasant Lake in Minnesota serves as an example of the usefulness of Fintrol-15 in deeper, thermally-stratified water. This lake, 80 feet deep, is managed

for rainbow trout, but in recent years redbelly dace, bluntnose minnows, fathead minnows, blacknose shiners, and golden shiners had become abundant and heavily infested with parasites. The application was made to take advantage of the fact that the minnows prefer the upper layer of warm water in summer whereas the trout remain in the deep colder layers. When the lake was well stratified in late summer, the upper layer of water--the epilimnion--was approximately 15 feet thick. This layer was treated with Fintrol-15 and the shallower water along shore with Fintrol-5 to attain 5 ppb of antimycin in this upper stratum of warm water. As far as could be determined by trapping, netting, and SCUBA diving, the minnows were eliminated from the lake. There was a minor kill of rainbow trout which were presumed to have moved into the epilimnion to feed. Thus, treatment of the epilimnion of Pleasant Lake at the time of maximum thermal stratification was effective, practical, and much more economical than treating the entire body of water.

Fintrol-30 releases its antimycin within the first 30 feet of depth as the sand carrier sinks to the bottom. Its performance in penetrating to depths in thermally stratified water to reach target fish was observed in Beauty Lake in Quebec. The objective was to remove northern pike, minnows, white suckers, pumpkinseeds, and yellow perch to restore native brook trout in the 44-acre lake.

Beauty Lake has a maximum depth of 42 feet, and much of the lake exceeds 20 feet in depth--the shorelines are steep. Total treatment of the basin was necessary because the target species may have been distributed through a wide range of depths. We applied 5 ppb of antimycin as Fintrol-30 by boat to obtain as even a distribution as possible.

Fintrol-30 effectively released its antimycin in the deeper waters of the thermocline and was apparent within 2 hours after application. Large numbers of dead and dying American smelt rose to the surface of the lake. This species customarily inhabits the deeper, colder waters; indeed, its presence in Beauty

Lake had never before been detected by fishery managers. Moreover, no live fish other than brown bullheads were taken during post-treatment netting in the lake. The operation was entirely successful in eradicating target fish.

Among these formulations, Fintrol-5 is registered and available to fishery managers. Fintrol-liquid, Fintrol-15, and Fintrol-30 may soon have registered labels. Other formulations may be developed, depending on the needs of fishery managers. A delayed-release formulation on sand--one which would release its toxicant only after the sand has sunk to the bottom--might be useful for control of lampreys, bullheads, and other bottom-dwelling fish.

DETOXIFICATION OF ANTIMYCIN

Laboratory and field tests confirmed that potassium permanganate can detoxify antimycin in water. The amount of permanganate depends on the concentration of toxicant, but the quantities, in general, are modest. However, we cannot recommend use of potassium where water is to be used for domestic or agricultural purposes or where fish are to be consumed by man because it has not been registered for this use by the U.S. Department of Agriculture.

The reclamation of Rathbone Creek at Cataract, Wis., required detoxification of antimycin. Several miles of the small, trout stream were treated with sufficient antimycin to maintain a concentration of 7.5 ppb for at least 8 hours in order to eliminate populations of minnows and suckers. Cataract Pond, a small impoundment, was the downstream limit of reclamation. It had been drained to low level before the treatment and refilled during treatment. Refilling only 18 hours before antimycin-laden water reached the spillway of the dam, the pond was treated with 2 ppm of potassium permanganate. The detoxification was quick and complete as shown by the fact that effluent water did not harm rainbow trout in live cages 500 feet downstream from Cataract Pond.

RECOMMENDATIONS FOR USE OF ANTIMYCIN OR OTHER FISH CONTROL AGENTS

The following considerations are essential to the successful reclamation of static or flowing waters.

- I. Define the problem in full detail.
 - A. What is the cause of the problem?
 - B. What are the effects?
 - C. Is the problem temporary or long-term?
- II. Assess the fish populations involved or present by netting, trapping, electro-fishing, or creel census.
- III. Define the target species and their pertinent characteristics.
- IV. Evaluate all possible approaches to a solution of the problem; include cost benefit ratios.
 - A. Biological or environmental controls.
 - B. Chemical controls:
 1. General toxicant.
 2. Selective toxicant.
 3. Total reclamation.
 4. Partial reclamation.
 - C. Other controls electrical, mechanical:
 1. Electrical or mechanical weirs, barriers.
 2. Dams, fishways.
 - D. Integrated controls--combinations of A, B, and C.
- V. Select a control.
 - A. Consider its specificity to the target fish and life stages involved.
 - B. Consider possible side effects on other fish, aquatic life, and environment.
 - C. Consider effects or influences on multiple uses of the water.

If a chemical control is selected, the following additional considerations are advised. They apply to any of the registered fish toxicants. See table 5 for recommended concentrations.

- I. Make several determinations of the following, preferably in both warm and cold seasons:

Item	Lake	Stream
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A. General characteristics:

1. Areasurface acressurface acres.
2. Depthav., contoursav., contours.
3. Volume.....acre-feetacre-feet.
4. Flows.....current patternc.f.s.
5. Velocity.....ft./sec.ft./sec.

Item	Lake	Stream
6. Bottom:		
Littoral.....	type	type.
Profundal.....	type	type.
7. Tributaries.....streams, springs.....streams, springs.		
8. Gradient.....ft./mi.		
9. Pool grade.....type, frequency.		
10. Riffle grade.....type, frequency.		
11. Vegetation.....type, species.....type, species.		
12. Basin, channel.....open, occluded.....open, occluded.		
13. Configuration.....map, photograph.....map, photograph.		
B. Water characteristics:		
1. Chemistry.....pH.....pH.		
Do.....dissolved oxygen.....dissolved oxygen.		
Do.....carbon dioxide.....carbon dioxide.		
Do.....total alkalinity.....total alkalinity.		
Do.....CO ₃ alkalinity.....CO ₃ alkalinity.		
Do.....HCO ₃ alkalinity.....HCO ₃ alkalinity.		
Do.....total hardness.....total hardness.		
2. Resistance.....ohms.....ohms.		
conductance.....mhos.....mhos.		
3. Temperature.....°F., °C.°F., °C.		
4. Color.....type.....type.		
5. Turbidity.....type, density.....type, density.		
6. Pollution.....type, source.....type, source.		

TABLE 5.--Guidelines for selecting concentrations of antimycin to control freshwater fish

	pH below 7.5		pH 7.5-8.5		pH above 8.5	
	Water Above 60°	Water Below 60°	Water Above 60°	Water Below 60°	Water Above 60°	Water Below 60°
<u>Sensitive fish</u> ¹ :						
Antimycin A in ppb.....	5.0	7.5	7.5	10.0	10.0	10.0
Fintrol-5 in ppm.....	0.5	0.75	0.75	1.0	1.0	1.0
Fintrol-5 in pounds per acre foot.....	1.4	2.0	2.0	2.8	2.8	2.8
<u>Resistant fish</u> ² :						
Antimycin A in ppb.....	15	20	20	25	25	25
Fintrol-5 in ppm.....	1.5	2.0	2.0	2.5	2.5	2.5
Fintrol-5 in pounds per acre foot.....	4.1	5.5	5.5	6.9	6.9	6.9

¹ Species such as gizzard shad, trout, pike, carp, minnows, suckers, sticklebacks, white bass, sunfish, perch, freshwater drum, and sculpins.

² Gar, bowfin, goldfish.

II. Correlate all data on target fish, on the lake or stream, and on water characteristics to aid in--

A. Selection of toxicant, formulation, and concentration for--

1. Total or partial reclamation.
2. General or selective control.

B. Selection of season and time for best application of control, with regard to--

1. Favorable temperature for chemical activity.
2. Favorable water chemistry for chemical activity.
3. Minimum conflicting uses of water.
4. Minimum interference by aquatic vegetation.
5. Limited vs. general distribution of target fish spawning congregations; preferred distribution in thermally-stratified water and life stages involved.
6. Favorable logistics to remote waters.

C. Selection of method for most effective distribution of toxicant (the distribution must be well planned, organized, and executed without interruption):

1. By hand--time, labor, costs.
2. By boat--time, labor, costs.
3. By aircraft--time, labor, costs.

D. Consider auxiliary tools to enhance effectiveness or economy of toxicant, e.g.--

1. Reduce interfering aquatic vegetation by prior application of herbicide.
2. Reduce turbidity with a flocculating material.
3. Manipulate water levels to concentrate fish, reduce water volume.
4. Modify the pH and chemistry of the water.

III. Confirm selection of toxicant, the formulation, and concentration for desired activity against target fish by lakeside or streamside bioassays, as follows:

A. Several concentrations of toxicant and durations of exposure which bracket the proposed application rate must be tested in the bioassays.

- B. The water in the bioassays must be from the target lake or stream.
- C. Temperatures in the bioassays must be representative of the target lake or stream.
- D. Target species from the target lake or stream are preferred in the bioassays.

IV. Adjust the type, formulation, and concentration of toxicant and the time of reclamation as indicated by results of the bioassays.

V. Set live cages containing target and other indicator species at various sites and depths in the target water at least 24 hours prior to the reclamation. Check their survival just before the reclamation and periodically thereafter. Replace dead fish with fresh lots of specimens to detect as accurately as possible the persistence and activity of the toxicant. A lot of fish must include at least 10 specimens of the same species and size. When a fresh lot of the most sensitive species survives 48 hours of exposure in the treated water, the toxicant is considered degraded or removed from the basin.

VI. Postpone or cancel the reclamation if any conditions in the target water or reclamation process have changed significantly since the bioassays were made. The risk of failure--always present--becomes too high when conditions have changed.

VII. Collect, enumerate, process, and bury dead fish promptly.

VIII. Make thorough assessment of the results of the reclamation, by--

A. Netting, trapping, electrofishing, or SCUBA.

B. Detect and evaluate side effects on any aquatic or terrestrial life.

IX. Relate results to objectives. Were the objectives attained?

X. Clean up the reclamation site; properly dispose of surplus toxicant and empty containers.

XI. Prepare full report of the reclamation for files and/or publication.

XII. Prepare and distribute an addendum report on the type and success of post-reclamation management of the fishery.

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CORRECTIONS for

A RESUME ON FIELD APPLICATIONS
OF ANTIMYCIN A TO CONTROL FISH

by Robert E. Lennon and Bernard L. Berger

Investigations in Fish Control No. 40
of the
Bureau of Sport Fisheries and Wildlife
U.S. Department of the Interior
May 1970

Tables 1, 3, and 4 were incorrect in this paper, but are corrected on the following sheets. Please insert these sheets in your copy.

Table 1.--Delete the following species.

Am El	American eel	<u>Anguilla rostrata</u>
Bk Ss	Brook silverside	<u>Labidesthes sicculus</u>
Bt RdHs	Blacktail redhorse	<u>Moxostoma poecilurum</u>
Fl SnFh	Flier sunfish	<u>Centrarchus macropterus</u>
Lk Cb Sk	Lake chub sucker	<u>Erimyzon sucetta</u>
Sh TpmW	Starhead topminnow	<u>Fundulus notti</u>
Sp SnFh	Spotted sunfish	<u>Lepomis punctatus</u>
Tl Sh	Taillight shiner	<u>Notropis maculatus</u>

Table 3 --Corrected test sites and species

Trial Site	Species
5	GrPl, GdSh, LnSh, WdSh, WmBs, RbSnFh, GrSnFh, BgSnFh, ReSnFh, HySnFh, LmBs,
33	RbTr, AtSm, BrTr, BrkTr, NoPk, GdSh, BcSh, BnSh, LnMw, WtSk, BrBlHd, PsSnFh, YwPh, MtSp
2-4	RbTr, AtSm, BrTr, BrkTr, CnPl, GdSh, CmSh, LnDc, CrCb, FlFh, WtSk, BrBlHd, PsSnFh, BgSnFh, SmBs, LmBs, YwPh
34	AmBrkLy, BrkTr, CnMdMw, WtSk, BrkSb, JhDr
9-11	RbTr, Cp, GdSh, SmBf, BmBf, BfHy, ChCtFh, WmBs, GrSnFh, BgSnFh, LmBs, BlkCr
45	SpGr, Bf, GzSd, Chm, Cp, Qb, WtSk, SmBf, WtCtFh, YwBlHd, ChCtFh, WmBs, GrSnFh, PsSnFh, BgSnFh, ReSnFh, SmBs, LmBs, YwPh
30	CnMdMw, NoPk, Cp, GdSh, CrCb, WtSk, RkBs, GrSnFh, BgSnFh, LmBs, BlkCr
12	NoPk, Cp, FhMw, BkBlHd, GrSnFh, BgSnFh, LmBs, BlkCr, YwPh
13	NoPk, Cp, FhMw, BkBlHd, BgSnFh, LmBs, BlkCr, YwPh
19	LmBs, BlkCr
6-8	Bf, RbTr, BrTr, BrkTr, Chm, Cp, GdSh, WtSk, BrBlHd, WtBs, RkBs, GrSnFh, PsSnFh, BgSnFh, SmBs, LmBs, BlkCr, YwPh, We, FwDm
46	KkSm, CtTr, RbTr, BrTr, BrkTr, SoRbDc, LnSk, WtSk
48	Cp
49	CmJk, RbTr, BrkTr, DyVnTr, Pm, LnDc, LnDc, LsSk, RsSh, TrSp

Table 3.--(continued)

15	RbTr, BrTr, CnMdMw, NoPk, Cp, GdSh, FhMw, WtSk, BkBlHd, ChCtFh, GrSnFh, PsSnFh, BgSnFh
16	BrkTr, FhMw, CrCb, IaDr
22	Bf, CnMdMw, NoPk, Cp, BnDc, LnDc, WtSk, BrkSb, IgSnFh
23	Cp
31	Cp, FhMw
32	RbTr, Cp, PsSnFh, BgSnFh, YwPh
14	RbTr, BrTr, StRr, BnDc, LnDc, CrCb, WtSk, NoHgSk, BrkSb, JhDr, MtSp
44	Cp, BkBlHd
52	Cp, FhMw, BgSnFh
1	AmBrkLy, LnGr, Bf, RbTr, NoPk, Gf, Cp, WtSk, BkBlHd, YwBlHd, GrSnFh, PsSnFh, BgSnFh, HySnFh, LmBs, BlkCr, YwPh, We FwDm, BrBlHd
39	AmBrkLy, RbTr, Cp, BnMw, FhMw, BnDc, CrCb, WtSk, BrkSb, PsSnFh, BgSnFh, LmBs, FtDr, JhDr, MtSp
17	PdFh, GzSd, Gf, Cp, EmBf, WtCtFh, BlCtFh, BkBlHd, YwBlHd, ChCtFh, TpMt, FhCtFh, ShMw, WmBs, GrSnFh, OsSf, BgSnFh, LmBs, BlkCr, M ₁ Fh
18	PdFh, Cp, GdSh, SmBf, EmBf, ChCtFh, WmBs, GrSnFh, OsSf, BgSnFh, LmBs
51	Cp

Table 4.--Corrected test sites and species

Trial Site	Species
24	NoPk, BnMw, BkBlHd, RkBs, BgSnFh, SmBs, YwPh
25	NoPk, GdSh, BnMw, BkBlHd, RkBs, SmBs, YwPh
26	NoPk, GdSh, BnMw, BkBlHd, RkBs, SmBs, YwPh
47	GdSh, BrBlHd, ChCtFh, MqFh, RbSnFh, OsSf, BgSnFh, ReSnFh, LmBs, WtCr
35	CmSh, ChCtFh, M ₁ Fh, GrSnFh, LmBs
50	GdSh, ChCtFh, WmBs, BgSnFh, LmBs, MqFh
21	Cp
37	Cp, BgSnFh, LmBs, BlkCr
36	GdSh, GrSnFh, BgSnFh, LmBs, ChCtFh
43	ChCtFh, BgSnFh
41	RbTr, SoRbDc, GdSh, BnSh, BnMw, BnDc, WtSk, BrkSb
20	Cp
38	GdSh, ChCtFh, GrSnFh
27-29	GdSh, WmBs, GrSnFh, BgSnFh, ChCtFh
42	GrSnFh, PsSnFh
40	GzSd, GrSnFh

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 38. Toxicity of 33NCS (3'-chloro-3-nitrosalicylanilide) to Freshwater Fish and Sea Lampreys, by Leif L. Marking, Everett L. King, Charles R. Walker, and John H. Howell.
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- 41. Identification of MS-222 Residues
in Selected Fish Tissues
by Thin Layer Chromatography**
- 42. Dynamics of MS-222 in the
Blood and Brain of Freshwater Fishes
During Anesthesia**
- 43. Effect of MS-222 on
Electrolyte and Water Content
in the Brain of Rainbow Trout**



United States Department of the Interior
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INVESTIGATIONS IN FISH CONTROL

41. Identification of MS-222 Residues in Selected Fish Tissues by Thin Layer Chromatography

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IDENTIFICATION OF MS-222 RESIDUES IN SELECTED FISH TISSUES BY THIN LAYER CHROMATOGRAPHY

By John L. Allen, Chemist,
Charles W. Luhning and Paul D. Harman, Physical Science Technicians
Bureau of Sport Fisheries and Wildlife
Southeastern Fish Control Laboratory, Warm Springs, Georgia

ABSTRACT.--MS-222, a commonly used fish anesthetic, reacts with the Bratton-Marshall reagents to form a wine-red dye. Residues of MS-222 determined by this reaction are not distinguished from other primary aromatic amines. Thin layer chromatography was used to identify MS-222 in the presence of background primary aromatic amines in fish muscle, brain, and blood. This method, in which the Bratton-Marshall reaction is used to visualize the spots, gave both the specificity of the Bratton-Marshall reaction for primary aromatic amines and the R_f of MS-222 as tools for identification of the residues. Recoveries of 25 to 60 percent were obtained in muscle samples spiked with 2 to 10 ppm of MS-222. Quantitative estimation was difficult in samples spiked with 2 ppm or less, but presence of MS-222 residues could be confirmed in samples spiked with as little as 0.2 ppm. Since the meta-aminobenzoate ester can be identified at these concentrations, this should be a useful ancillary or confirmatory method for determining the rate of disappearance of drug residues in fish flesh and obtaining data for clearance and registration of the anesthetic with the Food and Drug Administration.

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is used extensively as an anesthetic for fish. Walker and Schoettger (1967) described a method for quantitative determination of MS-222 residues in fish tissues using a modification of the Bratton-Marshall (1939) method for sulfonamides. MS-222, a primary aromatic amine, gives a wine-red color when reacted with the Bratton-Marshall reagents. Since other primary aromatic amines give the same color, a method for specific identification of MS-222 residues is needed.

Thin layer chromatography has been useful in identifying primary aromatic amines. This technique was used by Bican-Fister and Kajganovic (1963) to visualize sulfonamides on thin layer plates by spraying the plates with modified Bratton-Marshall reagents. We chose

to investigate the application of thin layer chromatography, using modified Bratton-Marshall reagents, to visualize MS-222 residues in fish tissue. This system offers both the specificity of the Bratton-Marshall reaction for primary aromatic amines and the R_f of MS-222 as tools for the identification of the compound.

METHODS AND MATERIALS

Experiments on operating parameters

Various solvent systems were investigated for possible use as developers of the chromatograms on silica gel chromatography sheets without fluorescent dye. When a solvent system of 2-percent methanol in benzene was

used, MS-222 gave an R_f of approximately 0.5. However, a substance occurring in certain untreated carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) tissues gave the same red color with about the same R_f as MS-222 when this system was employed. This problem was overcome by using a solvent system of 91 percent benzene, 4 percent acetic acid, and 5 percent ethyl ether. With this system MS-222 gave an R_f of about 0.45, while the interfering substance in carp and goldfish tissue did not migrate above the spotting line.

We used the diazo coupling agent of Bratton and Marshall (1939) to develop a red-colored azo dye in the MS-222 spot on the chromatogram. This diazotization reaction with MS-222 was described in detail by Walker and Schoettger (1967). We found that this coupling reagent in spray solution gave the darkest spot when 0.2-percent sodium nitrite in 1.5-percent hydrochloric acid was used. After the plates were sprayed with the acidic nitrite solution, they were dried in a stream of hot air to destroy the excess nitrous acid. A coupling reagent of 0.1-percent *N*-1-naphthylethylenediamine dihydrochloride in water was satisfactory.

The minimum time required for the extraction of MS-222 residues from fish muscle in a Soxhlet extractor was determined. Fish muscle samples fortified with MS-222 were analyzed after 4, 8, 12, 16, and 24 cycles. Extraction was essentially complete after 8 cycles.

Reagents and apparatus

Reagents and apparatus were as follows¹:

1. Methanol, reagent grade.
2. Petroleum ether, reagent grade.
3. Florisil, 100-200 mesh.
4. Alumina, neutral, Brockman activity 1, 80-200 mesh.
5. Developing solution: 91-percent benzene (pesticide grade), 4-percent acetic acid (reagent grade), and 5-percent ethyl ether (U.S.F.).

6. 0.2-percent sodium nitrite in 1.5-percent hydrochloric acid: Dissolve 0.20 g of sodium nitrite in 50 ml of water, add 1.5 ml of concentrated hydrochloric acid and dilute to 100 ml with distilled water. Make fresh daily.
7. 0.1-percent *N*-1-naphthylethylenediamine dihydrochloride: Dissolve 0.10 g in 100 ml of distilled water. Store in a dark container, refrigerate, and make fresh weekly.
8. Standard solution of MS-222: Dissolve 10.0 mg of MS-222 in 100 ml of methanol.
9. Tissue homogenizer.
10. Silica gel thin layer plates, Eastman Chromogram Sheet without fluorescent dye, 20 x 20 cm.
11. Micropipettes, 1, 5, and 10 μ l.
12. Chromatography tank, 4 by 8 by 9 inches, lined with absorbent paper.
13. Chromatographic column, 400 x 24 mm I.D. with sealed in, coarse fritted disk.
14. Soxhlet extraction apparatus, I.D. of extraction tube 30 mm ; 80 x 25 mm thimbles.
15. Teflon coated muffin pan.

Tissue collection

Blood samples are drawn from specimens by cardiac puncture. (Walker and Schoettger, 1967) with a heparinized syringe fitted with an 18- or 20-gage needle.

Other tissue samples are collected from fish after killing them by a blow on the head or by pithing. Brain samples are dissected out of the immobilized fish, and muscle samples are collected by filleting the fish.

Sample preparation and extraction

Blood samples are extracted by adding 0.5 ml blood to 9.5 ml methanol, mixed thoroughly and applied to the chromatographic column for cleanup.

Brain samples are extracted by homogenizing 1 g of brain, or the entire brain if it weighs less than 1 g with 5 ml of methanol. The homogenate is then placed in the chromatographic column for cleanup.

¹Reference to a company or product does not imply recommendation to the exclusion of others that may be suitable.

Muscle samples (5 g) are taken from a homogenate of the entire fillet, spread thin in the bottom of the muffin pan, and dried at 80°C. for 6 hours. Grind the dried tissue to a powder with a mortar and pestle, transfer to a Soxhlet extraction thimble, and wash with three 15-ml portions of petroleum ether (discard the washings). Air-dry the washed sample and extract with 70 ml of methanol in a Soxhlet for a minimum of 8 reflux cycles. After the final cycle, allow the upper portion of the extractor to fill, but not to siphon over. The extract and the remaining methanol in the boiling flask is ready for column chromatographic cleanup.

Cleanup of extracts

Prepare a 400-mm by 24-mm column by adding 2.5 cm of alumina followed by 7.5 cm of Florisil. Tap the column gently to pack the adsorbent. Prewash the column with 50 ml of methanol. When the methanol wash just sinks into the surface of the column, add the sample extract to the column and begin collecting the eluate in a 100-ml beaker. Discard the methanol prewash. Rinse the flask which contained the extract 3 times with 2-ml portions of methanol, adding each consecutive rinse to the column just as the previous rinse disappears into the surface of the column. As the last of the methanol rinse sinks into the column surface, add 50 ml of methanol and collect the eluate only until the last of the methanol has disappeared into the surface of the column.

Use a hot water bath and a stream of dry air to concentrate the eluate to 3 to 5 ml. Quantitatively transfer the eluate to a 15-ml graduated centrifuge tube with methanol. Place the centrifuge tube in a hot water bath and concentrate the eluate to 0.5 ml under a stream of dry air.

Thin layer chromatography

Mark a spotting line 2.5 cm, and a solvent-front line 12.5 cm, from the bottom of an 8-by 8-inch thin layer plate.

When thin layer chromatography is employed to confirm the presence of MS-222 residues,

as determined by the method of Walker and Schoettger (1967), spot a volume of extract equivalent to 0.5 μ g of MS-222. To compensate for interferences inherent in the colorimetric procedure, spot 100 μ l of extract from samples containing 2.0 ppm or less of MS-222 residue.

When screening unknown samples which may contain MS-222 residues, a maximum of 100 μ l of sample extract is spotted on the spotting line. On the same line, spot 50, 10, and 5 μ l of extract along with a series of MS-222 standards in the range of 0.1 μ g to 1.0 μ g.

Thirty minutes before developing the thin layer plate, pour 200 ml of developing solution into a chromatographic tank lined with absorbent paper. Place the thin layer plate in the tank and allow the developing solution to rise to the previously marked solvent-front line. Remove the plate from the tank, mark any deviations of the solvent front, and allow to air-dry in a horizontal position.

Spray the plate with the acidic nitrite solution until the plate is uniformly damp, wait 3 to 5 minutes, and dry in a stream of hot air. When the plate is completely dry, spray with 0.1-percent *N*-1-naphthylethylenediamine dihydrochloride solution until damp and dry immediately with hot air.

MS-222 is seen as a red spot. The amount of MS-222 can be estimated by comparing the intensity and size of the sample spot to the MS-222 standard spots. MS-222 standards must be run simultaneously with the samples so direct comparison of the R_f of standard and sample can be made on the same plate. After quantitative estimation is complete, store plate in a dark dry container.

RESULTS AND DISCUSSION

The Bratton-Marshall color reaction is specific for primary aromatic amines. Therefore, any naturally occurring primary aromatic amine or drug containing a primary aromatic amine group develops a color when treated with the Bratton-Marshall reagents.

The presence of low levels of MS-222 residue is difficult to ascertain by the modified Bratton-Marshall method of Walker and Schoettger (1967) because of the background readings obtained from the tissues being analyzed.

Thin layer chromatography separates MS-222 from nine other chemicals containing the primary aromatic amine group. The R_f values for 0.5- μ g spots of MS-222 and nine other compounds containing the primary aromatic amine group are shown in table 1. The comparison of R_f values must be made on the same plate since these values may vary between determinations.

The minimum level at which quantitative estimations can be made from MS-222 standard spots was found to be 0.1 μ g. The maximum amount of muscle extract that can be spotted on the thin layer plate was found to be approximately 100 μ l, which is equivalent to 1 g of tissue.

The efficiency of the method was evaluated by analyzing muscle samples from channel catfish (*Ictalurus punctatus*) spiked with 0.2 to 10.0 ppm of MS-222 (table 2). Recoveries of MS-222 from samples spiked with 2.0 to 10.0 ppm ranged from 25 to 60 percent. Quantitative estimation becomes difficult at residue levels of 2.0 ppm or less owing to the large amount of sample which must be spotted. When large amounts of samples are spotted, accurate quantitation is prevented by spreading of the spot caused by interfering fats and other extraneous materials. However, presence of MS-222 in muscle tissue was confirmed in samples spiked with as little as 0.2 ppm of MS-222. The samples were spiked by injecting a methanol solution of MS-222 standard into the samples before they were oven-dried.

The method was effective for eliminating background interferences in the analysis of muscle tissue from 8 species of fish (table 3). A red spot was noted only in goldfish and carp, but it did not migrate above the spotting line.

Brain and blood samples from three channel catfish treated with 100 ppm of MS-222 to deep anesthesia were analyzed by thin layer chromatography after 0-hour, 1/2-hour, and 1-hour

Table 1.-- R_f values for MS-222 and nine other compounds which produce a red color by the MS-222 thin layer chromatographic method when the 91 percent benzene, 4 percent acetic acid, and 5 percent ethyl ether developing solution was used and 5 μ l of a 100-ppm solution of each amine was spotted

Compound	R_f value	R_{MS-222}^1
Benzocaine.....	0.48	1.17
MS-222 (tricaine methanesulfonate).....	0.41	1.00
Aniline.....	0.35	0.85
p-Aminobenzoic acid.....	0.27	0.66
m-Aminobenzoic acid.....	0.16	0.39
Sulfamerazine.....	0.07	0.17
Sulfamethazine.....	0.07	0.17
p-Aminohippuric acid.....	0.00	0.00
Penicillin G, procaine ²	0.00	0.00
Sulfanilic acid.....	0.00	0.00

$$^1 R_{MS-222} = \frac{R_f \text{ of sample}}{R_f \text{ of MS-222 standard}}$$

² Penicillin G, procaine, 300,000 u/cc.

Table 2.--Estimated recovery of MS-222 spiked into 5-g samples of channel catfish muscle as determined by the thin layer chromatographic method

Concentration of spike	Number of fish	Equivalent amount of muscle spotted ¹ (g)	Tr trace		
			Estimated amount of MS-222 detected (μ g)	Estimated concentration of MS-222 (ppm)	Percent recovery
Control.....	4	1.00	0.0	0.0	--
0.2 ppm.....	4	1.00	Tr	Tr	Tr-60
0.5 ppm.....	4	1.00	Tr-0.3	Tr-0.3	Tr-60
1.0 ppm.....	3	0.50-0.75	Tr-0.6	Tr-0.6	Tr-80
2.0 ppm.....	4	0.50-1.00	0.5-0.6	0.5-0.6	25-30
5.0 ppm.....	4	0.10	0.2-0.3	2.0-3.0	40-60
10.0 ppm.....	4	0.05	0.2-0.3	4.0-6.0	40-60

¹ High and low amounts of tissue spotted do not necessarily coincide with high and low amounts of MS-222 detected.

Table 3.--Analyses of eight species of untreated fish for the presence of naturally occurring interferences by the thin layer chromatographic method for MS-222

Species	Equivalent amount of muscle spotted (g)	Red spot detected	R_f of spot	R_{MS-222}
Northern pike, <i>Esox lucius</i> ...	1.0	No	--	--
Muskellunge, <i>Esox masquinongy</i> ...	1.0	No	--	--
Goldfish, <i>Carassius auratus</i> ...	1.0	Yes	0.00	¹ 0.00
Carp, <i>Cyprinus carpio</i>	1.0	Yes	0.00	¹ 0.00
Channel catfish, <i>Ictalurus punctatus</i>	1.0	No	--	--
Bluegill, <i>Lepomis macrochirus</i>	1.0	No	--	--
Largemouth bass, <i>Micropterus salmoides</i>	1.0	No	--	--
Walleye, <i>Stizostedion vitreum vitreum</i>	1.0	No	--	--

$$^1 R_{MS-222} = \frac{R_f \text{ of sample}}{R_f \text{ of MS-222 standard}} = \frac{0.00}{0.41} = 0.00$$

withdrawals in fresh water. The cleanup on these samples was satisfactory for thin layer chromatography. No recoveries were run on these tissues as the cleanup procedure for blood and brain is identical to that of muscle

tissue. Residues of MS-222 were shown to be present in each sample of blood and brain by a red spot having the same R_f as the MS-222 standards.

Kidney and liver samples could not be analyzed for MS-222 residues by this method, because our cleanup procedure was not effective for these tissues.

Since we were able to effectively isolate, recover, and identify trace concentrations of meta-aminobenzoate ester, this should be a useful ancillary or confirmatory method for determining the rate of disappearance of residues in fish flesh. To obtain clearance and registration of MS-222 as an anesthetic with the Food and Drug Administration, we must

generate residue data by analytical methods of sufficient sensitivity and reliability with confirmation by an ancillary method.

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42. Dynamics of MS-222 in the Blood and Brain of Freshwater Fishes During Anesthesia

By Joseph B. Hunn



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DYNAMICS OF MS-222 IN THE BLOOD AND BRAIN OF FRESHWATER FISHES DURING ANESTHESIA

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ABSTRACT,--Eleven species of freshwater fishes were rapidly anesthetized in solutions of MS-222 containing from 100 to 1,000 milligrams of MS-222 per liter. MS-222 concentrations in blood and brain after 1 minute of exposure indicate that MS-222 rapidly diffuses across the gill and passes the blood-brain barrier. Evidence of metabolism of the drug was seen in the presence of acetylated MS-222 in the blood of all species studied. The concentration of free MS-222 in the brain increased with depth of anesthesia to loss of reflex and then either increased or declined slightly as the fish approached medullary collapse.

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is an effective fish anesthetic when administered by immersing fish in a solution or by spraying it on their gills (Schoettger, 1967). In either case, the route of entry is the gills. MS-222 is a lipid-soluble compound which is only 0.01-percent ionized at body pH (Maren, Embry, and Broder, 1968). This lipid solubility most likely accounts for its rapid diffusion across the gills.

Once the drug enters the bloodstream, it is distributed throughout the body. Although the site of action of MS-222 has not been established, it is thought to be in the brain. The blood-brain barrier in fish is known to exclude certain dyes, such as sulfonilic acid, from the cerebrospinal fluid (Rall, 1967). Preliminary investigations by Stenger and Maren (1968) indicate that MS-222 effectively crosses this barrier in the dogfish shark (Squalus acanthias). My studies were designed to extend this observation by measuring the rate of uptake of MS-222 in blood and brain of freshwater fish during the induction of anesthesia.

METHODS AND MATERIALS

Eleven species of fish were obtained from several sources (table 1). All specimens were maintained according to the methods of Hunn, Schoettger, and Whealdon (1968), except carp

Table 1.--Sources and sizes of fish used in the MS-222 uptake studies

Species	Length (inches)	Weight (grams)	Source
Shortnose gar <u>Lepisosteus platostomus</u>	21.5-27.5	--	Mississippi River Guttenberg, Iowa
Longnose gar <u>Lepisosteus osseus</u>	27.0-33.5	--	Mississippi River Guttenberg, Iowa
Bowfin <u>Amia calva</u>	23.0-31.0	--	Mississippi River Guttenberg, Iowa
Rainbow trout <u>Salmo gairdneri</u>	11.0-16.0	296-720	National Fish Hatchery Manchester, Iowa
Northern pike <u>Esox lucius</u>	10.0-18.0	--	Mead Wildlife Area Marshfield, Wis.
Carp <u>Cyprinus carpio</u>	8.5-10.8	140-350	Mississippi River Genoa, Wis.
Spotted sucker <u>Minytrema melanops</u>	11.8-14.3	--	Mississippi River Guttenberg, Iowa
Black bullhead <u>Ictalurus melas</u>	7.0-9.6	84-220	Mead Wildlife Area Marshfield, Wis.
Channel catfish <u>Ictalurus punctatus</u>	9.8-15.8	104-540	Mississippi River Lansing, Iowa
White bass <u>Ambloplites rupestris</u>	12.2-14.0	435-620	Mississippi River Guttenberg, Iowa
Bluegill <u>Lepomis macrochirus</u>	7.5-9.3	197-345	National Fish Hatchery Fairport, Iowa

and black bullheads, which were held at 17°C. The anesthetic solution of MS-222 in well water was made up fresh daily. The MS-222 was technical grade (99.4 percent) methane-sulfonate of *m*-aminobenzoic acid ethyl ester obtained from Sandoz Pharmaceuticals. Desired concentrations of the drug were achieved by adding the crystalline material to measured volumes of well water in 5-gallon stainless-steel pails or in 45- or 100-liter polyethylene tanks. Individual fish were immersed in the anesthetic solution for periods of 1, 3, 5, 8, or 11 minutes. All fish were anesthetized to loss of reflex, and most were nearing medullary collapse in 8 to 11 minutes of exposure.

Blood samples were taken by caudal puncture (Steucke and Schoettger, 1967). The spinal cord of the fish was then severed and the brain removed. Concentrations of MS-222 and background primary aromatic amines in whole blood and brain were determined by the Bratton-Marshall method as modified by Walker and Schoettger (1967). The average concentration of background amines was sub-

tracted from total aromatic amines to determine the concentration of MS-222.

RESULTS

MS-222 moves rapidly across the gills and enters the bloodstream of fishes (table 2). Within 1 minute of exposure, the drug concentration greatly exceeds the background level of primary aromatic amines. The ratio of the highest average concentration of MS-222 in whole blood to that of the anesthetic solution ranged from 0.14 in shortnose gar to 0.83 in rainbow trout.

Background primary aromatic amines in whole blood ranged from 0.6 to 5.4 milligrams per liter (mg/l) as free amines, and 0.0 to 4.0 mg/l as acetylated amines.

In seven of the eleven species, the brain concentration of MS-222 exceeded that of the whole blood after the first minute of exposure (table 2). The brains of all species contained amounts of MS-222 in excess of those in the blood after 3 minutes.

Table 2.--Concentration of MS-222 in whole blood and brain of 11 species of fish during the induction of anesthesia
[Condition of anesthesia for each species listed in table 3]

Species and exposure time	Concentration in ppm									Brain-blood ratio
	Whole blood						Brain			
	Free MS-222			Acetylated MS-222			Free MS-222			
	n	Mean	Range	n	Mean	Range	n	Mean	Range	
Shortnose gar:										
0 min. ¹	2	0.6	0.6	2	1.8	1.0-2.6	2	3.6	3.2-4.0	6.0
1 min.....	2	143.2	93.8-192.6	2	10.3	0.0-20.6	2	135.6	128.8-142.4	0.95
3 min.....	2	135.0	135.0	2	8.2	7.8-8.6	2	342.8	301.6-384.0	2.54
5 min.....	2	108.2	105.4-111.0	2	8.8	8.6-9.0	2	269.6	238.4-300.8	2.49
8 min.....	2	122.8	95.0-150.6	2	0.9	0.0-1.8	2	230.4	190.8-270.0	1.88
Longnose gar:										
0 min. ¹	2	2.1	1.6-2.6	2	1.2	0.6-1.8	2	2.9	2.4-3.4	1.38
1 min.....	1	74.7	-	1	2.8	-	2	88.3	58.8-117.8	1.18
3 min.....	2	135.3	126.4-144.2	2	8.5	0.0-17.0	2	265.1	221.6-308.6	1.96
5 min.....	2	114.3	110.0-118.6	2	1.7	0.0-3.4	2	277.5	262.4-292.6	2.43
8 min.....	2	127.1	124.8-129.4	2	8.0	5.8-10.2	2	228.7	225.6-231.8	1.80
Bowfin:										
0 min. ¹	2	3.9	3.2-4.6	2	0.2	0.0-0.4	2	8.6	7.2-10.0	2.21
1 min.....	2	202.2	163.6-240.8	2	105.1	75.0-135.2	2	58.3	45.6-71.0	0.29
3 min.....	2	178.5	166.6-190.4	2	64.0	6.6-121.4	2	217.3	157.8-276.8	1.22
5 min.....	2	186.0	184.6-187.4	2	37.0	34.2-39.8	2	188.8	184.8-192.8	1.02
8 min.....	2	117.3	100.0-134.6	2	0.0	-	2	222.8	192.8-252.8	1.90

See footnotes at end of table.

Table 2.--Concentration of MS-222 in whole blood and brain of 11 species of fish during the induction of anesthesia--Continued

Species and exposure time	Concentration in ppm									Brain-blood ratio
	Whole blood						Brain			
	Free MS-222			Acetylated MS-222			Free MS-222			
	n	Mean	Range	n	Mean	Range	n	Mean	Range	
Rainbow trout:										
0 min ¹	8	1.7	1.3-2.8	8	0.7	0.4-1.1	12	3.2	2.3-5.6	1.83
1 min.....	4	69.2	43.3-104.2	4	3.8	0.6-7.9	8	116.0	107.7-125.7	1.68
2 min.....	5	51.9	42.7-66.8	5	3.1	0.6-6.9	8	145.1	136.8-150.7	2.80
4 min.....	5	68.6	49.9-82.2	5	1.7	0.0-5.3	8	165.4	159.1-169.2	2.41
6 min.....	4	68.5	60.9-72.6	4	2.0	0.4-3.1	5	156.8	146.1-172.0	2.29
10 min.....	5	83.1	66.3-94.2	5	2.9	0.0-4.9	5	154.1	144.9-159.6	1.85
Northern pike:										
0 min ¹	6	1.9	1.0-2.4	5	0.7	0.4-4.0	²³	1.8	1.6-2.2	0.95
1 min.....	6	35.9	23.0-57.6	6	1.5	0.0-3.9	²³	66.9	60.6-78.4	1.86
3 min.....	6	81.9	60.4-111.6	5	3.4	1.3-5.3	²³	152.9	140.8-158.6	1.87
5 min.....	6	86.0	73.0-100.4	5	13.1	4.0-16.8	²³	204.6	183.6-223.8	2.38
8 min.....	6	95.7	83.0-105.6	6	7.7	2.4-11.2	²³	248.1	230.4-257.8	2.59
Carp:										
0 min ¹	6	1.4	1.2-2.0	6	0.7	0.0-2.8	²³	1.1	1.0-1.4	0.79
1 min.....	6	63.9	34.4-82.0	6	3.8	0.0-17.2	²³	54.6	44.2-66.6	0.85
3 min.....	6	96.0	78.4-114.0	6	2.5	1.4-2.8	²³	164.1	155.8-171.4	1.71
5 min.....	6	90.2	82.8-97.8	6	17.2	3.4-29.8	²³	165.8	151.4-192.6	1.84
8 min.....	6	100.8	88.8-115.6	5	1.7	0.0-2.6	²³	190.0	187.0-192.6	1.88
11 min.....	6	89.5	72.4-105.0	6	3.5	0.0-6.3	²³	156.5	134.6-176.2	1.75
Spotted sucker:										
0 min. ¹	6	1.8	1.6-2.2	6	0.0	-	²³	4.9	4.0-6.6	2.7
1 min.....	2	113.4	86.2-140.6	2	0.0	-	^{2p}	35.5	-	0.3
3 min.....	2	100.3	66.0-134.6	2	10.7	7.0-14.4	^{2p}	143.7	-	1.4
5 min.....	2	108.9	58.8-159.0	2	6.6	6.4-6.8	P	190.0	-	1.7
8 min.....	2	121.0	103.8-138.2	2	0.7	0.0-1.4	P	200.3	-	1.6
11 min.....	2	102.7	83.2-122.2	2	1.5	1.0-2.0	P	184.1	-	1.8
Black bullhead:										
0 min. ¹	8	2.3	1.6-3.0	8	0.2	0.0-0.6	²⁴	4.8	4.0-5.5	2.09
1 min.....	6	68.8	42.0-148.0	6	0.4	0.0-2.8	²³	111.3	102.8-119.6	1.62
3 min.....	5	121.5	102.6-174.2	4	23.1	0.0-62.2	²³	162.8	140.3-185.7	1.34
5 min.....	6	145.5	109.0-197.0	5	19.3	12.0-27.4	²³	227.1	194.4-259.7	1.56
8 min.....	6	146.8	130.4-165.8	6	10.9	1.2-15.4	²³	266.9	252.0-284.5	1.82
11 min.....	6	127.9	110.0-156.2	6	11.1	8.0-15.4	²³	240.5	220.0-250.5	1.88
Channel catfish:										
0 min. ¹	16	1.2	0.6-1.8	16	1.2	0.6-1.6	8	3.9	2.6-4.6	3.25
1 min.....	4	114.4	104.4-120.6	4	3.2	0.0-8.2	4	188.6	178.1-198.1	1.65
3 min.....	4	137.3	123.8-164.0	4	11.7	6.2-14.2	4	260.3	239.7-290.5	1.89
5 min.....	4	116.4	99.8-120.4	4	11.4	0.8-19.6	4	235.6	227.7-243.7	2.02
8 min.....	4	106.7	94.0-111.0	4	7.6	2.2-9.8	3	182.3	150.7-240.2	1.71
11 min.....	4	115.2	104.2-140.8	4	9.2	5.6-13.2	4	217.9	184.5-258.6	1.89
White bass:										
0 min. ¹	3	4.1	3.4-5.4	3	0.4	0.0-1.2	3	2.7	2.0-3.8	0.65
1 min.....	2	42.8	39.7-45.9	2	3.8	3.6-4.0	2	47.7	45.1-50.3	1.11
3 min.....	5	86.7	44.3-102.5	5	10.1	2.8-14.4	5	109.7	77.1-132.9	1.27
5 min.....	2	90.3	84.7-95.9	2	21.2	14.8-27.6	2	115.1	104.9-125.3	1.27
8 min.....	2	88.9	86.3-91.5	2	13.8	12.8-14.8	2	130.9	125.3-136.5	1.47
Bluegill:										
0 min. ¹	6	1.9	1.4-2.6	6	0.9	0.0-2.2	²³	3.1	2.8-3.2	1.61
1 min.....	6	62.7	27.6-93.4	6	2.9	0.0-8.6	²³	89.0	67.6-109.6	1.42
3 min.....	6	104.3	87.2-123.0	5	5.1	0.0-9.2	²³	168.2	152.4-189.2	1.61
5 min.....	12	121.8	87.7-134.1	11	12.8	0.0-63.9	²⁶	174.2	129.3-206.5	1.43
8 min.....	6	98.5	91.0-104.6	6	17.5	12.0-20.6	²³	196.5	180.0-208.0	1.99
11 min.....	6	95.5	74.2-117.0	6	8.7	0.0-14.6	²³	174.2	163.6-179.6	1.82

¹Background level of primary aromatic amines.^{2p} = pooled sample, 2 brains per sample.

Table 3.--Concentrations of MS-222 in blood and brain of 11 species of fish at loss of reflex stage of anesthesia

Species	Temperature °C.	Anesthetic concentration (mg/l)	Time in anesthetic at loss of reflex (minutes)	Average concentration of free MS-222 ¹	
				In whole blood (mg/l)	In brain (mg/kg)
Shortnose gar.....	12	1,000	2-3	135.0	342.8
Longnose gar.....	12	800	2-3	135.0	265.1
Bowfin.....	12	1,000	2-3	178.0	217.3
Rainbow trout.....	12	100	3-4	68.6	165.4
Northern pike.....	12	150	2-3	81.9	152.9
Carp.....	17	200	3-4	96.0	164.1
Spotted sucker.....	12	200	2-3	100.3	143.7
Black bullhead.....	17	200	5-6	145.5	227.1
Channel catfish.....	12	200	2-3	137.3	260.3
White bass.....	12	150	2-3	83.6	107.1
Bluegill.....	12	200	2-3	104.3	168.2

¹ Average concentrations of free MS-222 compiled from table 2.

A minimum concentration of 100 milligrams per kilogram (mg/kg) of free MS-222 appears to be necessary for anesthesia to loss of reflex judging from the average concentrations measured in the brain of 11 species (table 3).

DISCUSSION

Diffusion of MS-222, a highly lipid-soluble nonpolar drug, across the gills of fish is quite rapid. Movement of the drug may be in either direction depending on the concentration gradient. This study has shown concentrations of MS-222 in both blood and brain greatly in excess of background amines after 1 minute of exposure to the anesthetic solution. As shown by Maren, Embry, and Broder (1968) in their study on the dogfish shark, the gill is quite efficient in clearing the blood of MS-222 during recovery from anesthesia. Hunn, Schoettger, and Willford (1968) have indirectly measured the same phenomenon in rainbow trout. Preliminary investigations by Maren, Broder, and Stenger (1968) showed that the nonpolar ethyl *m*-aminobenzoate and its *N*-acetyl derivative are both excreted across the gill while the polar *m*-aminobenzoic acid and its *N*-acetyl derivative are excreted via the kidney. Most of the MS-222 and its congeners are excreted via the gills during recovery; 95 percent in the dogfish shark (Maren, Embry, and Broder, 1968) and 79 to 85 percent in the rainbow trout (Hunn, Schoettger, and Willford, 1968).

Concentrations of MS-222 in whole blood (table 2) drawn via caudal puncture did not reach the levels in the anesthetic solutions during exposures as long as 11 minutes (fish approaching medullary collapse). This is probably due to the fact that blood drawn by this method is usually venous blood which would contain a lesser concentration of the drug than arterial blood until the drug is in equilibrium between the fish and the anesthetic solution.

The appearance of acetylated MS-222 in most blood samples indicates that all 11 species are able to metabolize it. Highest concentrations of acetylated drug were usually detected after 3 to 5 minutes of exposure. The bowfin had the greatest blood concentration of acetylated MS-222 of any of the 11 species studied, 34 percent after a 1-minute exposure. Concentrations in the 10 species were usually less than 20 percent. Maren, Broder, and Stenger (1968) found the same level of acetylated drug in the plasma of the dogfish shark during recovery from anesthesia.

Stenger and Maren (1968) reported that during MS-222 anesthesia of the dogfish shark, the drug rapidly reaches the cerebrospinal fluid and the brain. My observations confirm this finding. In all 11 species, the concentration of free MS-222 in the brain was significantly above background after 1 minute of exposure. The concentration of drug in the brain

increased with depth of anesthesia to loss of reflex. With deeper anesthesia, the concentration of free MS-222 either increased slightly or declined in comparison with the concentration at loss of reflex. A concentration of at least 100 mg/kg is necessary for anesthesia to loss of reflex in susceptible species like rainbow trout, whereas the more resistant species like black bullhead require approximately 200 mg/kg of the free drug for a similar level of anesthesia.

In a previous paper (Hunn, 1968) I noted that rapid recovery in fresh water is associated with the declining concentration of free MS-222 in the brain of channel catfish. The brain concentration of free drug was 91.6 mg/kg when the catfish righted themselves whereas it was 260.3 mg/kg when they exhibited loss of reflex. Indeed, in all studies published to date anesthesia and recovery in fresh water have been strictly associated with the concentration of free drug in the blood and brain (Schoettger et al., 1967; Walker and Schoettger, 1967b).

SUMMARY

Eleven species of freshwater fish were rapidly anesthetized in solutions of MS-222 containing from 100 to 1,000 mg/l of drug. MS-222 (free and acetylated) concentrations in whole blood and brain after 1 minute of exposure indicate that MS-222 rapidly diffuses across the gill and passes the blood-brain barrier. Blood samples drawn by caudal puncture contained lower concentrations of MS-222 than those of the anesthetic solutions.

The presence of acetylated MS-222 in the blood of all species studied is evidence that fish metabolize the drug. Concentrations of acetylated drug were usually less than 20 percent of the total MS-222 except those in bowfin which had 34-percent acetylation after a 1-minute exposure.

MS-222 rapidly enters the brain from the blood. The concentration in the brain increases with depth of anesthesia to loss of reflex. As fish enter more deeply into anes-

thesia, the drug concentration either increases slightly or declines in ratio to the levels at loss of reflex. Anesthesia and recovery in fresh water appears to be associated with the concentration of free MS-222 in the blood and brain.

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43. Effect of MS-222 on Electrolyte and Water Content in the Brain of Rainbow Trout

By Wayne A. Willford



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EFFECT OF MS-222 ON ELECTROLYTE AND WATER CONTENT IN THE BRAIN OF RAINBOW TROUT

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ABSTRACT.--Rainbow trout (*Salmo gairdneri*) were exposed to 100-milligrams-per-liter solutions of MS-222 for 1-, 2-, 4-, and 10-minute intervals and their brains were analyzed for sodium, potassium, calcium, magnesium, zinc, iron, and water content. The mean potassium content decreased 17.7 percent and iron increased 56.2 percent during 2-minute exposures. Sodium and calcium increased slightly (7.4 and 9.4 percent); magnesium, zinc, and water content remained relatively constant. All of the affected electrolytes returned toward control values with 4- and 10-minute exposures. These shifts in electrolytes appear to be related to depth of anesthesia and to the concentrations of free MS-222 in the brain.

MS-222 (methanesulfonate of meta-amino-benzoic acid ethyl ester) has been used extensively as an anesthetic for coldblooded animals including fish and amphibians (Schoettger, 1967). However, little is known about its mode of action.

It is generally believed that anesthetics act by absorption or combination with lipid groups in the cell membrane and somehow alter the cell membrane's function of establishing ionic gradients and regulating respiratory rates in cells (Skou, 1961; Quastel, 1963). The result is disruption of ionic differential, the biopotential and ratios by which nerve impulses are propagated. In addition, upsetting the ionic equilibriums may further affect the rates of reactions which restore standing biopotentials (Hillman, 1966).

Walsh and Schopp (1966) demonstrated that MS-222 and related compounds reduce the frequency of electric organ discharges in the electric fish (*Gnathonemus moorii*). They concluded that these agents apparently inhibit pacemaker cells in the mesencephalic command nucleus. Stenger and Maren (1968) and Hunn (1970) have further shown that MS-222

rapidly crosses the gill of fish and is concentrated in the brain. The depth of anesthesia was associated with the concentration of MS-222 in the brain.

The objective of this investigation was to detect and measure changes in brain electrolytes which are associated with MS-222 anesthesia. The electrolytes chosen for study were those which appear essential to the production and maintenance of nerve potential and metabolic activity.

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*) were obtained from the National Fish Hatchery, Manchester, Iowa. The fish ranged in weight from 390 to 720 grams and were delivered in two shipments during the 6 months of testing. Each group of fish was held in flowing well water at 12° C. and was fed on a diet of commercial trout pellets supplemented with liver.

The test fish were placed individually into 5 liters of well water containing 100 mg/l (milligrams per liter) of MS-222 for 1, 2, 4 or 10

minutes. This concentration of anesthetic produces deep anesthesia in rainbow trout within 3 minutes and medullary collapse in approximately 10 minutes (Schoettger and Julin, 1967).

After exposure, the fish were removed from the anesthetic and decapitated. Whole brains were removed carefully to avoid contamination and were blotted dry and placed in tared porcelain crucibles. After determination of wet weight, the samples were dried to constant weight at 95° C. Brains of control fish were excised and processed in the same manner.

All samples were dry-ashed according to the approved method for lead using 1 milliliter of 15.5 N nitric acid as the "ash-aid" (Horwitz, 1960). After ashing, 2 ml of 12.1 N hydrochloric acid were added to each crucible, and the resulting solution was concentrated to approximately 1 ml. The concentrate was quantitatively transferred by multiple rinses with distilled, deionized water into a 10-ml volumetric flask containing 1 ml of a 5-percent lanthanum solution in 25-percent (V/V) hydrochloric acid. The lanthanum chloride reduces chemical interference during analysis (Elwell and Gidley, 1966).

The samples were analyzed for sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), magnesium (Mg²⁺), zinc (Zn²⁺), and iron (Fe³⁺) on an atomic absorption spectrometer. Standard curves were prepared from composite standard solutions of all six elements in the presence of 0.5-percent lanthanum and 10-percent (V/V) hydrochloric acid.

The experiment was performed six times over a 6-month period using three fish for each of the four exposure intervals, and three fish for controls. The data for each brain constituent were analyzed statistically using a two-way analysis of variance to determine the significance ($p \leq 0.05$) of observed changes (Snedecor, 1956).

RESULTS

Anesthesia of rainbow trout in 100 mg/l of MS-222 at 12° C. resulted in significant shifts ($p < 0.005$) of K⁺ and Fe³⁺ concentrations in the

brain (table 1). There was a 17.7-percent reduction in K⁺ and a 56.2-percent increase in Fe³⁺ during the initial 2 minutes of exposure as determined by comparison with the controls. With longer exposures, the concentrations of K⁺ and Fe³⁺ returned toward control values.

Minor, nonsignificant ($p > 0.25$), increases of 7.4 and 9.4 percent were observed in the concentrations of Na⁺ and Ca²⁺ respectively in the brains of MS-222 anesthetized trout. Though these shifts were not significant, they do suggest an effect of the anesthetic which is similar to that observed in Fe³⁺. The significance of these measurements may have been masked by indeterminate variation.

The Mg²⁺, Zn²⁺, and water contents of the brains remained relatively constant over the entire range of exposures.

In addition to the changes observed during anesthesia, significant variation ($p = 0.025$ to < 0.005) of all the cations and the water content occurred between monthly replicates. The monthly variation in control fish appeared to be random, and resulted in diverse levels of

Table 1.--Brain cation and water content of rainbow trout exposed to 100 mg/l of MS-222 at 12° C. for selected intervals of time

Constituent	Exposed for--				
	Unexposed	1 minute	2 minutes	4 minutes	10 minutes
Na ⁺ mg/kg ¹	2 8,978 ±1,160	9,370 ±1,589	9,646 ±1,119	9,650 ±1,092	9,514 ±1,147
K ⁺ *	14,260 ±1,890	13,140 ±1,850	11,740 ±2,330	13,720 ±2,140	13,920 ±2,310
Ca ²⁺ mg/kg	612.6 ±68.2	656.9 ±115.1	670.2 ±120.4	649.7 ±94.4	625.3 ±99.3
Mg ²⁺ mg/kg	570.0 ±37.3	593.4 ±55.9	584.2 ±32.4	578.6 ±33.1	576.7 ±47.4
Zn ²⁺ mg/kg	47.82 ±5.39	47.00 ±7.63	49.48 ±14.03	48.97 ±9.35	47.48 ±9.10
Fe ³⁺ *	79.65 ±13.45	104.06 ±29.98	124.38 ±40.46	119.13 ±43.40	103.24 ±33.61
H ₂ O g/100 g	81.43 ±0.57	81.26 ±0.82	81.14 ±0.73	81.28 ±0.77	81.41 ±0.62

¹ Concentration of all cations based on dry tissue weight.

² Mean ± standard deviation (n=18).

*Significant variance attributable to exposure ($p < 0.005$).

Table 2.--Monthly variation of brain cation and water content of rainbow trout not exposed to MS-222

Constituent	Sampled on--					
	11/16/67	12/11/67	1/16/68	2/12/68	3/21/68	5/6/68
Na ⁺ mg/kg ¹	² 8,333 ±1,032	7,839 ±128	8,451 ±901	10,353 ±370	9,945 ±1,001	8,952 ±1,081
K ⁺ mg/kg	12,380 ±3,110	14,990 ±460	14,260 ±680	15,570 ±880	12,950 ±1,800	15,410 ±1,690
Ca ²⁺ mg/kg	617.3 ±61.7	549.2 ±31.4	581.9 ±100.1	641.5 ±32.7	596.0 ±51.1	690.3 ±53.8
Mg ²⁺ mg/kg	571.0 ±32.2	512.8 ±11.9	566.3 ±29.4	596.3 ±12.1	561.8 ±26.6	612.3 ±12.1
Zn ²⁺ mg/kg	51.30 ±3.81	47.43 ±3.88	52.39 ±9.29	43.61 ±1.87	44.27 ±0.44	47.92 ±6.02
Fe ³⁺ mg/kg	75.59 ±17.41	80.04 ±3.80	69.06 ±10.94	78.22 ±10.00	82.43 ±6.12	92.57 ±22.66
H ₂ O g/100 g	80.99 ±0.67	81.35 ±0.54	81.45 ±0.26	81.58 ±0.75	82.08 ±0.42	81.13 ±0.39

¹ Concentration of all cations based on dry tissue weight.² Mean ± standard deviation (N=3).

ions and water in the brain from replicate to replicate (table 2). However, the shift in ions attributable to MS-222 exposure (table 1) was similar within each replicate.

DISCUSSION

The results of this study show that MS-222 disrupts, directly or indirectly, specific cationic equilibria in the brain of rainbow trout during anesthesia. Disruption of ionic differentials, such as K⁺/Ca²⁺ and K⁺/Na⁺ ratios, has a profound effect on nerve potentials and respiration, this being the basis of the general theory of anesthetic action (Quastel, 1963; Hillman, 1966).

A major decrease in brain K⁺, however, is not peculiar to anesthesia. Systemic stress such as anoxia, heat, and cold also produce similar cationic imbalance in fish and mammals in vivo (Benjamin, Anastasi, and Helvey, 1961a; Hickman et al., 1964; Van Harreveld, 1966; Bandurski, Bradstreet, and Scholander, 1968). Benjamin, Anastasi, and Helvey, (1961b) have further shown by in vitro studies with rat brain that temperature does not directly effect K⁺ release but both anoxia and lack of glucose do. Since MS-222 reduces the respiratory rate of fish (Campbell and Davis, 1963; Randall, Smith, and Brett, (1965), an associated anoxia or hypoglycemia may have contributed to the K⁺ depression which I observed.

Unlike the progressive K⁺ loss that reaches a plateau during systemic stress, MS-222-induced anesthesia causes an initial K⁺ decrease during 1- and 2-minute exposures followed by a return toward control levels up to the time when the fish is approaching death at 10 minutes. The Fe³⁺ change is the reverse of this pattern.

Hunn (1970) showed that a similar pattern occurs in the concentration of free MS-222 in the brain of rainbow trout during anesthesia. The concentration of MS-222 increases rapidly during the first 2 to 4 minutes of exposure and then slowly declines with longer exposures. The exposure period at which the cation and MS-222 concentrations reverse direction of change coincides with the approximate exposure which produces loss of reflex in the fish. Electrolyte content of the brain appears to be associated with the MS-222 concentration in the brain and the depth of anesthesia.

Hillman (1966) stated that in nearly all conditions examined in mammals, changes in the K⁺ content of brain are mirrored in opposite changes in the Na⁺ content. In this study, as previously mentioned, the K⁺ decrease is mirrored by a marked increase in the Fe³⁺ content and only a slight increase in the Na⁺ content. Possibly this phenomenon can be explained in light of the work by Germain and Gagnon (1968). They demonstrated that the blood of hagfish (*Myxine glutinosa* L.) accumulates in subcutaneous sinuses during MS-222 anesthesia. They postulated that this accumulation was due to reduced respiratory movements and profound changes in hemodynamics during narcosis.

Since the brain is a relatively vascular tissue (Zwehl, 1961; Heisey, 1968), it is possible that a pooling or plasma skimming which concentrates erythrocytes, may occur in the brain during anesthesia. This would tend to mask changes in Na⁺ and K⁺ concentrations and cause an increase in the Fe³⁺ content of the brain.

SUMMARY

1. Anesthetizing rainbow trout in 100 mg/l of MS-222 at 12° C. for 2 minutes causes a

significant reduction of K^+ and increase of Fe^{3+} in the brain. A concurrent minor increase in the Na^+ and Ca^{2+} of the brain is observed.

2. Exposure of the fish to MS-222 for periods longer than 2 minutes results in a return of K^+ , Fe^{3+} , Na^+ , and Ca^{2+} towards control values. This pattern of change appears to be associated with the concentration of free MS-222 in the brain and with the depth of anesthesia.
3. MS-222 anesthesia of rainbow trout does not affect the concentrations of Mg^{2+} , Zn^{2+} , or water in the brain.
4. Whereas shifts in ions appear to be closely associated with anesthesia, it is possible that the large increase in Fe^{3+} may be a secondary effect due to erythrocyte pooling in the brain.
5. Significant monthly variation in the electrolyte and water content of brain occurs in rainbow trout held under the conditions described.

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44. A Review of Literature on TFM
(3-trifluormethyl-4-nitrophenol)
as a Lamprey Larvicide



United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife



**44. A Review of Literature on TFM
(3-trifluormethyl-4-nitrophenol)
as a Lamprey Larvicide**

By Rosalie A. Schnick



**United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife**

Washington, D.C. • April 1972

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A REVIEW OF LITERATURE ON TFM (3-trifluormethyl-4-nitrophenol) AS A LAMPREY LARVICIDE

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Abstract. --The search for a selective toxicant to control the sea lamprey, scourge of Great Lakes fisheries, culminated in the discovery of TFM (3-trifluormethyl-4-nitrophenol) in the late 1950's. The research, however, had only begun on its chemical and physical properties, efficacy, toxicity to non-target and target species, residues, and methods. Federal, State, university, and industrial agencies contributed much in the effort to determine the effect of TFM on the environment and other organisms; however, much still remains to be done if TFM is to gain continued clearance by the Environmental Protection Agency for use as a larval lampri-
cide in the aquatic environment.

HISTORY OF LAMPRICIDE DEVELOPMENT

The United States Congress directed the Fish and Wildlife Service in 1946 to develop measures for the control of the exotic sea lamprey^{1/} in the Great Lakes (Van Oosten, 1949a). A master plan was prepared, and a Sea Lamprey Committee was organized by Dr. John Van Oosten, Chief of the Great Lakes Fishery Investigations. One objective of the plan was a study of sea lamprey physiology to determine what agents, including chemicals, might be used to kill the lamprey in any life stage (Van Oosten, 1949b).

Congress appropriated funds for research in 1949 (U. S. Committee on Merchant Marine and Fisheries, 1951). The Fish and Wildlife Service in 1950 took over the U. S. Coast Guard Station at Hammond Bay, Michigan, on the northwest coast of Lake Huron, and renovated the

facilities for research on mechanical, electrical, and chemical controls for the sea lamprey (Moffett, 1950).

The initial studies to find toxicants selective to the sea lamprey were assigned to Philip J. Sawyer, a doctoral candidate at the University of Michigan on a Fish and Wildlife Service Research Fellowship (Applegate, 1950; U. S. Branch of Fishery Biology, 1952). Sawyer experimented with 179 compounds at the University before his project was terminated in 1952 (Applegate *et al.*, 1952). Six of the compounds that were selectively toxic to larval lampreys had nitro groups and through hydrolysis yielded a nitrophenol (Sawyer, 1956). Significantly, the chemical group later considered effective against lamprey were the mononitrophenols of which TFM is a member. In the meantime, the Michigan Conservation Department had determined that rotenone was not effective on sea lamprey larvae in streams (U. S. Committee on Merchant Marine and Fisheries, 1952).

^{1/} Petromyzon marinus. See Appendix B for common and scientific names of fishes listed in the text.

Following its early emphasis on mechanical and electrical controls for the sea

lamprey, the Hammond Bay Laboratory began screening up to 50 chemicals a day in 1953 (Applegate, 1953; U. S. Branch of Fishery Biology, 1953). Over 4,000 selected compounds tested under a military contract at the Fish and Wildlife Service's Microbiological Laboratory at Leetown, West Virginia, were transferred to Hammond Bay for trials against lampreys (Applegate *et al.*, 1957; U. S. Branch of Fishery Biology, 1953). These compounds had been bioassayed against four species of fish and separated into three groups of very toxic, moderately toxic, and negative compounds (Woods, 1953; Lennon, 1954). An additional 2,000 chemicals were obtained from universities, industries, and private individuals (McVeigh, 1958). The results of tests on compounds obtained from the National Academy of Sciences-National Research Council were reported in Summary Tables of Biological Tests (U. S. Fish and Wildlife Service, 1956a and 1956b).

While the U. S. Fish and Wildlife Service was looking for a selective larvicide, investigators in Canada attempted to find a general toxicant that would kill lamprey without too much harm to other fish populations, and would be inexpensive, available, easy to use and handle, and nontoxic to warm-blooded animals (Kerr, 1954). Although they found that toxaphene killed 70 percent of the lamprey larvae, it was too toxic to other fish and warm-blooded animals (Johnson and Tibbles, 1962).

Throughout 1954 and 1955 efforts at Hammond Bay centered almost exclusively on testing a large number of compounds (Applegate, 1954 and 1955). Finally, in 1955, of the eight compounds selectively toxic to sea lamprey, two proved to be promising (Applegate, 1955). One was Compound No. 174, 3-bromo-4-nitrophenol, originally from the Chemical-Biological Coordination Center and the Leetown testing program (Applegate, 1955; Wood, 1953). The other was Compound

No. 3579. The restricted Compound No. 3579 and a Pennsalt chemical, O-ethyl-S-pentachlorophenyl thiolcarbonate, may be one and the same for various reasons. A patent for lampricidal use of O-ethyl-S-pentachlorophenyl thiolcarbonate was applied for as early as December 29, 1955 (Neumoyer, 1960). Both compounds at 1 to 3 ppm (parts per million) at 55° F caused 100 percent mortalities among sea lamprey without affecting other fish (Applegate, 1955, 1958, and 1963). Both compounds were difficult to formulate and apply (Applegate, 1955; Howell, 1966). Raceway tests were still made on O-ethyl-S-pentachlorophenyl thiolcarbonate in 1957, but the compound was dropped from the program shortly thereafter (U. S. Bureau of Commercial Fisheries, 1957a; Howell, 1966).

Applegate and Howell (1960) applied for a patent on Compound No. 174, 3-bromo-4-nitrophenol, on March 29, 1956. Whereas 3-bromo-4-nitrophenol was soluble enough in water for larvicidal use, it was hard to synthesize and too expensive to use (Willeford, 1956; McVeigh, 1958; Moffett, 1958a; Howell, 1966). Thus in 1956, the investigators at Hammond Bay turned their attention to other nitrophenols.

In response to a request, at least six companies supplied many nitrophenols (Applegate, Howell, and Smith, 1958). Of the six most promising compounds tested during 1956, five were nitrophenols and one was NP-1458, a Pennsalt chemical (U. S. Fish and Wildlife Service, 1956c and 1956d; Applegate *et al.*, 1957). By April 11, 1957, six mononitrophenols showed enough promise that Applegate and Howell (1965) applied for a United States patent on control of the sea lamprey with mononitrophenols. On March 18, 1958, Applegate and Howell (1964) applied for a Canadian patent on the same compounds. Pyne (1962) later applied for a British patent on one of the mononitrophenols, 2,5-dichloro-4-nitrophenol. Testing in the field demonstrated that

nitrophenols are stable, measurable by a colorimeter, usable under various water quality conditions, harmless to domestic animals and wildlife at the concentrations used, not too toxic to other fish and invertebrates, and inexpensive enough to warrant further research (Moffett, 1958a).

Among nine compounds suggested by Dr. Clarence L. Moyle of Dow Chemical Corporation was one, 3, 4, 6-trichloro-2-nitrophenol, that showed promise (McVeigh, 1958). It was tested against lamprey larvae in raceways, for acute oral and dermal toxicity to mammals, and in drinking water for deer and dairy cows (U. S. Bureau of Commercial Fisheries, 1957a and 1957b). Finally, the compound was tested in the field against larval sea lampreys. On October 29 to 30, 1957, Dowlap 30 (30 percent stock solution of 3, 4, 6-trichloro-2-nitrophenol as a sodium salt), was applied to Little Billies Creek (Elliot Creek), Michigan, resulting in a 96.6 percent kill of lampreys and little harm to other fauna (Moffett, 1958a and 1958b; Westerman, 1958; U. S. Bureau of Commercial Fisheries, 1957a and 1957b). Further testing was done in 1958 on Carp Creek, (Black Mallard Creek), Michigan, with Dowlap 20 (20 ppm of 3, 4, 6-trichloro-2-nitrophenol), but the results disclosed that Dowlap 20 or 30 had to be used in relatively high concentrations (13 ppm) and did not have a high differential in selectivity (Anonymous, 1959; Keller, 1966; U. S. Bureau of Commercial Fisheries, 1958a and 1958b).

The search continued, and promising compounds were tested both in the laboratory and raceways (Moffett, 1958a; U. S. Bureau of Commercial Fisheries, 1957a). A compound more effective at lower concentrations than Dowlap 30 was found - TFM (Moffett, 1958a).^{2/} "It was formu-

lated as a sodium salt, dissolved in an acceptable solvent, and sold to the Fish and Wildlife Service as Lamprecid 2770 by the Progressive Color and Chemical Company of New York" (Moffett, 1958a). Another source listed TFM as originally produced by Farbwerke Hoechst of Germany as a dye intermediate and supplied by the U. S. outlet, Hoechst Chemical, under the name of Lamprecid 2770 (Anonymous, 1959). When the U. S. Bureau of Commercial Fisheries originally began dealing with Farbwerke Hoechst of Germany, the American outlet was the Progressive Color and Chemical Company and later the Hoechst Chemical Company. Farbwerke Hoechst Aktiengesellschaft later succeeded in obtaining patents on TFM for lampricidal use in the United States, Great Britain, Germany, and Canada (Scherer, Frensch, and Stähler, 1960, 1962, and 1964).

Because TFM killed lamprey at 2 ppm, it was easier and cheaper to transport than Dowlap 30 (Keller, 1966; Johnson, 1961). Therefore, the U. S. Government purchased 10,000 pounds of TFM from Dow Chemical Company in 1958, 25,000 pounds from Maumee Chemical Company in 1959, and 25,000 pounds for use in 1960 from Dow Chemical Company (Anonymous, 1959; U. S. Bureau of Commercial Fisheries, 1959b). The Canadian unit obtained the amine salt of TFM through the Maumee Chemical Company for use in 1960 and 1961 treatments (Tibbles et al., 1961).

The first field testing of TFM was done on the Mosquito River, Michigan, May 14, 1958, and on the Silver River, Michigan, June 11, 1958 (Moffett, 1958a). The trials were successful, and they marked the end of research efforts in the field. Control crews treated eight streams with two formulations in 1958. One compound contained 45 percent active ingredient (Lamprecid 2770), and the other contained 30 percent active ingredient (Dowlap F40) (U. S. Bureau of Commercial Fisheries, 1958b). The

^{2/} 3-trifluormethyl-4-nitrophenol. See Appendix A for other names and technical data for TFM.

experimental field work on the Canadian side was completed on the Pancake River, Ontario, August 26 to 27, 1958, and on the West Davignon River, Ontario, November 5, 1958 (Johnson and Tibbles, 1962; Johnson, 1959; Fisheries Research Board of Canada, 1958). Checks were made on the effects of TFM on invertebrates and fish. In all these tests, TFM performed well enough to cause the Great Lakes Fishery Commission to authorize treatment of streams for control of sea lamprey (Moffett, 1958a).

PHYSICAL AND CHEMICAL PROPERTIES

The identification of TFM was accomplished in 1930. In the preparation and isolation of nitrotrifluoracetanilide, DeBrouwer (1930) obtained a substance which he identified as 1, 3, 6-nitrotrifluorocresol, or TFM. He determined the melting point (76°), molecular weight (207), position of the NO_2 grouping, resistance of the radical CF_3 , the constant of ionization (4.4×10^{-7}), and the preparation of its barium salt.

Applegate *et al.* (1961) listed several properties of TFM including its form at room temperature, color, solubility, stability, detoxification, and properties in different formulations. Smith, Applegate, and Johnson (1960) determined that TFM is a fairly strong acid ($\text{pK } 6.07 \pm 0.03$), that its color is at its height at pH 8.0 or above, and that it has a molar absorbance of 13,130 at 395 nm (nanometer). They also determined its ultraviolet, infrared, and visible spectra, and potentiometric titrations (Smith, Applegate, and Johnson, 1961). Daniels *et al.*, (1965) mentioned the molecular weight, melting point of the free phenol and the impurities of TFM. Rogers and Watson (1968) studied the electron spin resonance (esr) spectra of the anion radicals of TFM.

In an attempt to determine the reason for the selectivity of mononitrophenols, especially TFM, Applegate, Johnson, and Smith (1966) tested phenols with nitro groups in the *para* position and the halogen in the *meta* position and discovered that they are generally more toxic to lampreys. Other related compounds were studied for possible selective properties, but none was found. A condensed version of the preceding report appeared a year later (Applegate, Smith, and Willeford, 1967).

Observations at present seem to indicate that the toxicity of TFM is related to the amount of free phenol present. When TFM is exposed to low pH (6.8), it exists as a free phenol, but when exposed to high pH, most of the TFM exists as a phenolate ion (Lennon, 1971).

Information on the degradation and residues appears in the section of this report entitled, Residues.

Several methods for preparing TFM are known. Maumee Chemical Company used benzotrifluoride as a starting point (Anonymous, 1959). In the preparation of 2, 5-dihydroxybenzotrifluoride, Whalley (1949) found one of the derivatives to be 2-nitro-5-hydroxybenzotrifluoride ($\text{C}_7\text{H}_4\text{F}_3\text{NO}_3$) or TFM. The preliminary laboratory tests were done with a sample recrystallized from benzene-petroleum ether (Applegate *et al.*, 1961). Using 3-(trifluormethyl)-4-nitro-aniline, a diazonium was formed which was then hydrolyzed in a series of steps to yield TFM (U. S. Rubber Company, 1963). None of the various methods has produced a very pure compound. Farbwerke Hoechst produced a technical-grade preparation (91 percent pure) of TFM, which was used in raceway tests and all experimental stream treatments (Johnson, 1959 and Applegate *et al.*, 1961). Lech (1971) discussed methods for crystallizing TFM, and for preparing reduced TFM

(RTFM) and tritiated TFM and RTFM. Synthesis of C¹⁴ ring-labeled TFM was accomplished by the Mallinckrodt Chemical Works (1971).

EFFICACY

In preliminary laboratory tests at 55±1° F, a 2 ppm concentration of TFM with both free phenol and sodium salts was effective on sea lamprey larvae (Applegate, Howell, and Smith, 1958). In preliminary field tests, an application of 5.5 ppm of TFM for 9 hours killed all lamprey larvae within 7-3/4 hours in the Mosquito River, Michigan, and a concentration of 2.8 ppm applied for 13-1/2 hours killed all lamprey larvae in the Silver River, Michigan (U. S. Bureau of Commerical Fisheries, 1958b). A range of 3 to 10 ppm was established for lamprey control in laboratory tests by 1962 (Applegate and King, 1962). Taking various water chemistries into account, concentrations of 1 to 17 ppm are lethal to lamprey larvae (Dykstra and Lennon, 1966).

Life stages

Applegate, Howell, and Smith (1958) found that TFM was effective against sea lamprey in the 2- to 5-year classes at 2 ppm. Later, Applegate et al. (1961) exposed larval, recently transformed, and adult lampreys to 1.5 to 13 ppm of TFM in raceway tests at 45 to 55° F. The mortality of larvae was 100 percent at concentrations as low as 3 ppm. At 1.5 ppm, 91.5 percent of the larvae died. Adults and recently transformed lampreys died at concentrations as low as 3 ppm. Thus, treatment of streams when the adults are spawning in spring, or when the recently transformed lampreys are moving downstream in fall or winter could destroy some of the migrants. Dustin and Higginson (1967) observed that larvae in late stages of transformation (mid-summer) seem to be more resistant to a mixture of TFM

and Bayluscide[®] (a synergist used to reduce the amount of TFM needed).

Piavis (1962) undertook a study in 1956 to determine whether certain selective larvicides would have any effect upon the developing embryos, prolarvae (larvae still bearing a yolk), and young larvae. He found that all Stage 18 larvae (larvae with gut instead of yolk) exposed to 10 ppm of TFM died in 1 or 2 hours. It seemed to Piavis that chemical treatment of earlier stages (blastula through hatching) delayed development and led to death, but that prolarvae could survive until they become larvae. He suggested, therefore, that control of the sea lamprey would be most effective if conducted at least 40 days after all spawning so that larvae would have reached Stage 18 in their development.

In 1962, investigators at Hammond Bay exposed sea lamprey at all stages of embryonic development to 10 ppm of TFM for 24 hours. After exposure, eggs and larvae were washed and placed in fresh water. The exposure of Stages 1 through pre-hatching Stage 13 resulted in disintegration of the embryo before it reached Stage 18. All embryos exposed during prolarval stages, 14 to 17, died during or immediately following exposure. All Stage 18 larvae died within hours after exposure (Erkkila, 1962).

Temperature

Preliminary work on the effects of temperature on the efficacy of TFM was done in the laboratory. Lamprey larvae from 3.5 to 5 inches long were exposed to TFM in water from Hammond Bay of Lake Huron at 35, 45, and 55° F. The results indicated little difference in the activity of TFM at these temperatures. The MLC (minimum lethal concentration - the concentration killing 100 percent of the larvae within 24 hours) for larvae was 2 ppm, and the death rate slowed as the temperature decreased. The selectivity in toxicity between fish and

lamprey increased when the temperature dropped near 32° F (Applegate et al., 1961; Keller, 1966).

When the use of TFM was extended to include warm-water streams, a graduate student at the University of Michigan tested American brook lamprey from 3.5 to 7.5 inches long at 55, 60, and 65° F in Huron River water, and at 35 to 75° F (10-degree intervals from 35 to 55°, and 5-degree intervals from 55 to 75°) in city water (Cooper, 1965). In both waters the time required for effective exposure increased at colder temperatures. He found that the minimum lethal concentration was 5 ppm at 55°, 4 ppm at 60°, but at 65° F it was again 5 ppm. Cooper suggested that: 1) results may differ if pre-control bioassays are done in water warmer than the stream; 2) less chemical is needed if treatments were postponed until streams warm up; and 3) large streams should not be treated in late fall, winter, or early spring. He concluded that lower temperatures slow the death of lamprey, and added that no data are available on the interaction of temperature and water quality.

The results observed in field operations support those obtained through research. One of the first reports of water temperature affecting the toxicity of TFM in the field was in connection with an incomplete kill of larval lamprey in the Sucker River, Michigan. Shortly after the application of TFM, the temperature dropped from 55 to 39° F, which apparently retarded the activity of the free phenol and resulted in inadequate exposure (U. S. Bureau of Commercial Fisheries, 1958b). A later report found one of the causes for lamprey surviving a treatment in the lower Brunsweiler, a tributary of the Bad River, Wisconsin, was a drop of 11° F in temperature which reduced the effectiveness of TFM (Smith and King, 1970). Workers also observed that winter treatments required too much chemical and resulted in mechanical

failure of equipment due to freezing (U. S. Bureau of Commercial Fisheries, 1961). In addition, winter treatments made traveling, sampling, and observations difficult because of the ice and snow (Tibbles et al., 1961).

Water chemistry

Water from 16 streams in the Lakes Huron, Michigan, and Superior watersheds was used in bioassays at 55° F in the laboratory to check the effects of pH, conductivity, alkalinity, and turbidity on TFM. The chemical is most effective in soft, acid waters where the minimum lethal concentration is as low as 0.5 ppm. The hardest and most alkaline waters require 8 ppm. Turbidity caused no change in toxicity in a few simulated stream trials (Applegate et al., 1961). The dissolved oxygen was not checked because most streams to be treated have high oxygen concentrations.

The biological activity of TFM is generally best at about pH 7.1, but is reduced below 7.0 and as alkalinity increases. Attempts to change pH of streams have not been effective (U. S. Bureau of Commercial Fisheries, 1960b and 1960c). Lamprey larvae were exposed to six concentrations of TFM (1, 2, 3, 9, 11, and 13 ppm) for 20 hours at four pH values (6.6, 7.2, 7.8, and 8.4). All larvae died when exposed to 2 and 3 ppm of TFM at pH 6.6 to 7.8. Above pH 7.8, complete kills were not attained even at concentrations of 13 ppm (LeMaire, 1961).

In testing for comparative toxicity of TFM, Applegate and King (1962) selected water from three different sources which encompassed nearly all conditions in streams of Lakes Huron and Michigan. At pH 7.8, methyl orange alkalinity of 95.4 ppm, CO₂ of 1.6 ppm, and conductivity of 176.3 micromhos (at 18° C), the MLC of TFM was 4 ppm in 9 of 11 tests; at pH 7.7, methyl orange alkalinity of 141.7 ppm, CO₂ of 3.2 ppm, and conductivity of 219.1

micromhos, the MLC of TFM was 6 ppm in 6 of 11 tests, and at pH 7.7, methyl orange alkalinity of 203.3 ppm, CO_2 of 5.1 ppm, and conductivity of 338.7 micromhos, the MLC of TFM was 9 ppm in 5 of 10 tests. Thus, TFM is more toxic in waters having low alkalinity and conductivity.

Kanayama (1963) worked on a method to define the minimum lethal concentration of TFM. He measured alkalinity in tributaries of Lakes Superior and Michigan, and tested TFM at the various levels. The MLC of TFM ranged from 1 ppm at 10 to 19 ppm of CaCO_3 to 6.7 ppm at 160 to 169 ppm of CaCO_3 . He tested conductivity values from 40 to 279 micromhos (at 20° C), and found the MLC of TFM ranges from 1 ppm at 40 to 59 micromhos to 5.7 ppm at 260 to 279 micromhos. His results confirmed the fact that TFM is more toxic to lampreys at low levels of alkalinity and conductivity.

An investigation was made on the water quality of streams tributary to Lakes Superior and Michigan to determine the natural levels and seasonal fluctuations in concentrations of aluminum, copper, iron, magnesium, calcium, chloride, nitrate, nitrite, silica, sulfate, tannin-like and lignin-like compounds, phenolphthalein alkalinity, total alkalinity, total hardness, pH, and conductivity. This was done to facilitate determination of the minimum lethal concentrations of TFM to larval lamprey (Zimmerman, 1965; Smith, 1966).

A need to correlate these constituents with the biological activity of TFM has been demonstrated many times in field situations. Thus, investigators in Canada used water from various streams in bioassays, and analyzed the water at the same time. In addition to the constituents evaluated by Zimmerman (1965), they measured oxygen consumed, chemical oxygen demand, carbon dioxide, color, turbidity, suspended matter, residue on

evaporation, loss on ignition, manganese, zinc, sodium, potassium, ammonia, fluoride, and phosphate. No single constituent was linked consistently with the results of bioassays in the three streams tested; thus, no one factor was completely responsible for change in activity. More work needs to be done in this area (Johnson, 1970).

A few management experiences illustrate the problems related to differences in water chemistry. On one occasion when the concentration of dissolved oxygen in the Kalamazoo River, Michigan, was low (0.6 ppm to 2.4 ppm), 4 ppm of TFM and 2 percent Bayluscide® were required to get a complete kill of lamprey, but numbers of northern pike, carp, white sucker, and channel catfish were killed. In southern tributaries of Lake Michigan, 10 to 12 ppm of TFM were needed because of the water quality (Smith, 1967a). Manion (1969) found that effective concentrations of TFM range from 6 to 9 ppm and exposures range from 7 to 8 hours in waters of 4 to 10° C, 49 to 56 ppm of CaCO_3 , and 109 to 113 micromhos.

Natural factors

Certain physical and meteorological conditions can reduce the effectiveness of TFM treatments. Lamprey control crews have observed the following: 1) dilution of chemical concentration caused by excessive rain, merging streams, melting snow, and other volumetric increases; 2) high and low water levels interfere with distribution of the toxicant; 3) unless the sample is filtered, turbidity makes measurement of concentration difficult; 4) any naturally occurring substance or pollutant absorbing light at 395 nm may interfere with the determination of TFM; 5) escape of lampreys from TFM by burrowing deeper into the mud or traveling into untreated areas; 6) variation in chemical characteristics of the stream water, especially pH, which changes the biological activity of TFM; and 7) natural formations such as beaver dams which obstruct dispersion of the chemical (U. S. Bureau of

Commercial Fisheries, 1959a and 1961; Smith and King, 1969; Smith 1967a; Fisheries Research Board of Canada, 1959).

Management results

Control of the sea lamprey has been the greatest fish control endeavor ever attempted (Bardach, 1964). The success of this program is evidenced in the number of self-sustaining populations of game and food fish which are not seriously threatened by lamprey predation (Baldwin, 1968). Great recoveries of valuable fish stocks have been noted in Lake Michigan and Lake Superior (Crowe, 1965; Lennon et al., 1970; Tody, 1966; Wiegert, 1966). Observations by experts attribute this success to the reduction in numbers of sea lamprey to 10 percent of their pre-control abundance (Lawrie, 1970; Anonymous, 1967).

Indications are that the sea lamprey will be reduced further by improved survey and treatment techniques and by complete treatment of lamprey-producing tributaries of all Great Lakes. The total benefits to be derived from the control program greatly exceed the cost of research and management that must continue in order to suppress and maintain the sea lamprey at low numbers (Brinser et al., 1968; Lawrie, 1970; Lennon et al., 1970).

TOXICITY-- NON-TARGET SPECIES

Aquatic plants

At the concentrations used, TFM does not appear to affect adversely either algae or higher aquatic plants (Howell, 1966). However, TFM and its salts applied at concentrations from 15 to 100 ppm control rooted aquatic plants (Josephs, 1961). The lower doses are used in standing waters while the higher concentrations are needed

in moving waters. Each concentration requires about 2 hours of exposure. TFM and its salts and esters in liquid or dust form have also been used to protect seeds and make them resistant to attack by organisms causing damping off, seed rot, and root rot (Baker, 1962).

Haas (1970) treated streams with 1 and 4 ppm of TFM to determine the effect on periphyton. He weighed the standing crops before and after treatment, and found no significant difference in the growth rates of the two groups.

Invertebrates

Scherer, Stähler, and Frensch (1957) and Schrader (1961) studied the toxicity of the phosphoric acid ester of TFM to the common house fly, Musca domestica. They found that this compound is nontoxic to warm-blooded animals.

Scherer, Frensch, and Stähler (1960 and 1964) observed during field studies that lower aquatic organisms such as leeches (Hemiclepsis spp. and Glossiphonia spp.) and tubificids are affected at 3 to 7.5 mg/l (milligrams per liter) of TFM. On the other hand, fish-food forms such as Daphnidae, Coleoptera, Odonata, Notonectidae, and Gammaridae are not affected by TFM until concentrations reach 20 to 24 mg/l.

The Great Lakes Biological Laboratory (1963) reported a German study on the effect of TFM on invertebrates. This study determined that animals with a strong exoskeleton are not affected by a concentration of 10 ppm for 5 days. Gammarids tolerated 8 ppm and some insect larvae 6 ppm. Thin-shelled invertebrates died readily at the same concentrations. Leeches, Tubifex, and Daphnia tolerated 1.5, 3.75, and 3.75 to 4.0 ppm, respectively.

Applegate et al (1961) in their preliminary field tests of TFM noticed premature

emergence of mayfly nymphs, but treatments do not appear to seriously affect other groups of invertebrates.

To gain further knowledge on effects of TFM to common aquatic invertebrates, assays were completed with 14 groups representing five phyla and using 2 to 20 ppm of the toxicant for each species. Mortality was insignificant in exposures to 20 ppm among isopods, gammarids, crayfish, dragonflies, water boatmen, and case-building caddisflies. Concentrations below 10 ppm were harmless to leeches in the family Glossiphoniidae, stoneflies, bloodworms, and snails, but mortalities for this group were 10 to 55 percent at concentrations between 10 to 20 ppm. Mortality was significant with *Hydra* at 2 ppm, leeches (Hirudiniidae) at 8 ppm, burrowing mayflies at 6 ppm, net-building caddisflies at 13 ppm, blackflies at 3 ppm, and clams at 8 ppm (Erkkila, 1962). These results were much the same as those obtained in Germany, excluding the fact that the leeches tolerated 1.5 ppm there and 20 ppm here (Erkkila, 1964).

Smith (1967) tested invertebrates in the laboratory, and found that 100 percent of the hydras, turbellarians, and blackflies were affected when exposed to 10 ppm of TFM for an extended period. Other invertebrates such as burrowing mayflies would be reduced by 99 percent, Hirudiniidae by 89 percent and clams by 50 percent.

In the field, freshwater scud (*Gammarus* spp), burrowing mayflies, aquatic earthworms, and clams have been found dead in significant numbers after certain TFM treatments (U. S. Bureau of Commercial Fisheries, 1958b, 1959b, 1960b, and 1961).

In field tests in five tributaries of Lake Superior and four tributaries of Lake Michigan, Torblaa (1968) noted that one week after treatment 77 percent of the

invertebrate groups present in sand and detritus areas had decreased, 17 percent increased, and 6 percent showed no change. In riffle areas 64 percent had decreased, 19 percent increased, and 17 percent showed no change. In two untreated riffle areas 33 percent had decreased, 50 percent increased, and 17 percent showed no change. Elmidae and Helidae declined in sand areas, and Trichoptera, Coleoptera, Ephemeroptera, and Diptera declined in riffle areas. Six weeks after treatment, the numbers of organisms increased in three streams, were partially restored in another, and were reduced in one. In one year, complete recoveries were made (Smith, 1966).

Haas (1970) evaluated the effect of TFM on bottom fauna. He selected 12 taxa from riffle fauna and 8 taxa from pool fauna, and treated the stream with 1 ppm and 4 ppm of the lampricide. No change in riffle or pool fauna was observed from a TFM treatment at 1 ppm. All taxa, except two, decreased in numbers in the experimental riffle area, and all taxa decreased in the pool at 4 ppm of TFM. However, the bottom fauna varied greatly even without TFM, so it was not possible to determine the exact effect of TFM on bottom fauna.

TFM does not have molluscidal properties at levels used against sea lamprey, but at 15 mg/l for 6 hours the sodium salt of TFM (Eelicide--TFM) had a LC 99.5 rating (lethal concentration - concentration producing a 99.5 percent mortality within a specified period of time) for the snail (*Australorbis glabratus*) that is an intermediate host of schistosomiasis. At that level it was not toxic to the guppy and other small fish tested (Jobin and Unrau, 1967).

Fishes

European species were not affected by TFM until levels of 15 to 18 mg/l were reached (Scherer, Frensch, and Stahler, 1964). In preliminary tests in the United States TFM as a free phenol was toxic to

brown trout at 7 ppm and to rainbow trout at 9 ppm. Rainbow trout were affected at a 7 ppm concentration of the sodium salt of TFM (Applegate, Howell, and Smith, 1958; McKee and Wolf, 1963). Raceway studies included 15 species of fish, TFM at 1.5 to 13 ppm, and temperatures from 45 to 55° F. Brown trout, brook trout, and panfish tolerated TFM as well as did rainbow trout. Brown trout and rock bass were affected at a concentration of 9 ppm, adult white sucker and yellow perch above 7 ppm, logperch at 5 ppm, and bullheads above 3 ppm (Applegate et al., 1961).

Applegate et al (1961) observed in field trials that only a few species were affected adversely by TFM. Of 25 species of fish and four species of native lamprey in Silver River, Michigan, only logperch were killed in large numbers. Among 18 species of bony fishes only sculpins suffered great mortalities in the Pancake River system, Ontario.

Because lamprey control was extended to streams containing large numbers of warm-water fishes, toxic levels of TFM had to be established for bluntnose minnow, fathead minnow, white sucker, yellow bullhead, pumpkinseed, bluegill, smallmouth bass, largemouth bass, yellow perch, and walleye (U. S. Bureau of Commercial Fisheries, 1960a and 1960b). Lethal concentrations were established in three dilution waters for all of the above species except the bluntnose minnow and pumpkinseed. Tolerances also were established for golden shiner and blacknose shiner. TFM is as toxic to cyprinids as it is to rainbow trout. Centrarchids are more tolerant than the cyprinids. White sucker, yellow bullhead, yellow perch, and walleye are relatively susceptible, with the walleye the least tolerant. The MAC (maximum allowable concentration - concentration killing 25 percent of the specimens within 24 hours) ranges from 5 to 44 ppm depending upon the species of fishes present and the water quality

conditions at the time of application (Applegate and King, 1962).

Recent toxicity studies were performed at the Fish Control Laboratory, La Crosse, Wisconsin, on fingerling-size fish of 12 species. Using standard reconstituted water Marking (1971) found that the toxicity ranged from 1.39 to 16.2 ppm in 96-hour static exposures. Green sunfish and bluegill were the most resistant, and channel catfish were the most sensitive. The toxicity easily defined at 1, 3, and 6 hours of exposure. In tests to determine the effect of temperature on the biological activity of TFM, yellow perch were exposed to 12, 17, and 22° C, but very little difference was observed. On the other hand, in tests with rainbow trout TFM was found to be more toxic at 17° C than at 12 or 7° C (Lennon, 1971). Changes in toxicity did occur in different water qualities with TFM being more toxic in soft water. The LC50 for yellow perch ranges from 2.28 ppm in soft water (10 ppm total hardness) to 28.4 ppm in hard water (300 ppm total hardness) at 96-hour exposures. Also, TFM is more toxic to fish in water with low pH than high pH (Lennon, 1971).

In stream treatments several workers have observed fish mortalities when the water temperatures increased or changed drastically. A rapid rise in temperature of 8° F in the Muskegon River, Michigan, and variable temperatures in the Au Gres River, Michigan, possibly contributed to fish mortalities (Smith and King, 1969 and 1970). During the treatment of the Nottawasaga River, Ontario in June 1968, extreme water temperatures stressed the fish enough that normally safe TFM dosages caused fish mortalities (Lamsa, Dustin, and Davis, 1969; Davis and Shera, 1969).

The physiological effects of TFM on bony fishes differ in some respects from those in lamprey. Both bony fishes and lamprey accumulate a large amount of fluid in the tissue between the respiratory epithelium

and the vascular endothelium. Both exhibit mucous accumulation and vasodilation. However, their activity response differs in that lamprey become narcotized and hemorrhage, but in contrast rainbow trout surface, not hemorrhaging, but suffocating (Christie and Battle, 1963; Keller, 1966). Plasma concentrations of potassium, calcium, magnesium, lactic acid, and glucose increased, but sodium declined when adult white suckers were exposed to 5 ppm of TFM (Hunn, 1971; Lennon, 1971). Preliminary studies seem to indicate that the mode of action of TFM involves the release of potassium and the blocking of glucose utilization, resulting in increased plasma concentrations of lactic acid and glucose (Lennon, 1971).

Toxicity of TFM mixed with 2 percent of Bayluscide® to fishes changes somewhat, as compared to TFM alone. In the hard-water streams of Lake Michigan the mixture of toxicants increases the mortality of fish (Smith, 1967a; U. S. Bureau of Commercial Fisheries, 1965). If the amount of Bayluscide® is increased, the selectivity to lampreys over fish decreases (Smith, 1967b; Lamsa, 1968). In waters with high alkalinity, the toxicity of the mixture to fish increases (Smith, 1968).

Tests were performed on chinook salmon and coho salmon to determine the comparative effects of TFM and TFM with 1 percent of Bayluscide® (TFM-1B). The mixture was less toxic than TFM to both salmons. Coho salmon were affected less than chinook salmon by either compound. Care should be taken, however, not to treat streams when jack chinook salmon are running (Smith and King, 1969).

The toxicity of TFM to different fishes as reported from actual treatments varies according to conditions, but generally the species cited most as being affected by TFM are central mudminnow, white sucker, bullheads, stonecat, trout-perch, logperch, walleye, and sculpins. Occas-

ionally brown trout are susceptible, mainly because treatments are made in September and October when they are spawning (U. S. Bureau of Commercial Fisheries, 1959b and 1960b; Erkkila, 1964; Smith, 1967a; Johnson, 1959). Howell (1966) found that native lamprey of the genera Lampetra and Ichthyomyzon are affected almost as much as the sea lamprey in stream treatments.

Davis and Wilson (1965) compared the susceptibility of Ichthyomyzon spp. and American brook lamprey in field bioassays of TFM and TFM plus Bayluscide® at three exposure times on three rivers. The lethal concentrations using only TFM ranged from 0.9 to 4.8 ppm at 9 hours, 2.7 to 3.7 ppm at 12 hours, and 1.8 to 2.5 at 18 hours. Canadian investigators found that TFM is more toxic to sea lamprey than native lampreys; therefore, bioassays against native lampreys tend to indicate too high a concentration for safe use in streams (Great Lakes Fishery Commission, 1970; Davis, 1970). Furthermore, Davis (1970) contends that no simple correction factor is available which would provide accurate concentration determinations from tests with native lamprey genera.

Amphibians

There are no records of laboratory tests of TFM on amphibians. Observations in the field have shown that amphibians generally are not affected by TFM treatments in streams. In the post-treatment operations on the Pancake River in 1958, Johnson (1959) found dead only one each of tadpoles and Necturus. Howell (1966) states that Necturus maculosus seems to be as susceptible as lamprey to TFM. According to tests run in a 0.1-acre pond by the Fish Farming Experimental Station, Stuttgart, Arkansas, a complete kill of tadpoles is possible with an application of 12 ppm or more of TFM (Anonymous, 1964).

Reptiles

Small numbers of turtles were exposed to 3, 5, and 10 ppm at temperatures of 7.7, 7.2, and 6.4° C, respectively, causing no mortalities (Scherer, Frensch, and Stähler, 1960). Chrysemys picta and Chelydra serpentina were exposed to the same dosages of TFM at temperatures from 43 to 45° F with none dying (Applegate et al., 1961). No observations of dead reptiles have been recorded from the field.

Birds

No laboratory or field data exist for the effects of TFM on birds. Currently, the Denver Wildlife Research Center is investigating the oral toxicity of TFM to waterfowl and upland gamebirds (Lennon, 1971).

Mammals

Scherer, Frensch, and Stähler (1964) state that TFM, like other halogenated mononitrophenols, is toxic to warm-blooded animals at certain concentrations, and requires care in transporting and applying. Pure TFM has an oral LD50 (lethal dose that produces a 50 percent mortality within a specified period of time) of 30 mg/kg (milligram per kilogram) for the rat when injected intraperitoneally, but formulations consisting either of 50 percent of the sodium salt of TFM or 51 percent of TFM have LD50's of 300 mg/kg and 200 mg/kg to rats, respectively. According to Scherer, Frensch, and Stähler (1964), TFM in a free phenol formulation becomes very toxic orally and dermally.

Tests made by the Wisconsin Alumni Research Foundation on a 20 percent by weight formulation of TFM showed an acute oral LD50 for rabbit of 0.16 g/kg (grams per kilogram) and dermal LC50 of 1.6 g/kg. TFM is harmless to the

eyes at concentrations of 1 to 9 ppm but care should be taken in handling concentrated forms (Applegate et al., 1961; U. S. Bureau of Commercial Fisheries, 1957b).

Lech (1971) injected rats with TFM in 0.9 percent saline and found no signs of toxicity at 18 mg/kg, but toxic effects appeared at 24 mg/kg and mortality at 40 mg/kg.

The oral LD50 ranges recorded by Frear (1969) were from 0.5 to 1.0 g/kg of body weight for rats.

To determine the effects that TFM might have on mammals, the Michigan Conservation Department administered TFM in the drinking water given to four white-tail deer. According to the author, two deer had their water contaminated with the maximum of the chemical that would be used in the stream work. After 6 weeks no effects were observed although all four had drunk freely of treated and non-treated water (Johnson, 1957). Six 1,000-pound cows and four calves were exposed to water containing 13 ppm of TFM. Again no harmful effects were observed, and no phenol was detected in the milk of the animals (Applegate et al., 1961).

When higher concentrations of TFM up to 11 ppm were used in Lake Michigan tributaries, the Wisconsin Alumni Research Foundation found that they were not toxic to mammals (Erkkila, 1964).

In 1971, the WARF Institute, Inc. was awarded a contract to conduct acute and 90-day studies on the oral toxicity of TFM to laboratory animals (Lennon, 1971).

MODE OF ACTION ON SEA LAMPREY

The biological activity of TFM is affected by various factors which cause minimum lethal concentrations to range from 0.5 ppm to 12 ppm. The lower concentrations are

effective in soft, acid waters, and the higher doses are needed for hard, alkaline waters (Applegate et al., 1961; Scherer, Frensch, and Stähler, 1964; Applegate and King, 1962).

The physiological effects of TFM on the sea lamprey are not completely understood. The nervous tissue, cardiac musculature, notocord, alimentary canal, and mesonephros do not seem to be affected, but the gills, circulatory system, liver, and skeletal musculature are. In particular, the gills hemorrhage and become covered with mucous (Christie and Battle, 1963). Keller (1966) proposed that death was due to circulatory failure and suffocation. Applegate, Smith, and Willeford (1966) think lamprey die of a combination of circulatory and respiratory failure. One of the symptoms is a state of hypotension. The theory that death was due to anoxia was tested by Agris (1966 and 1967). He found that the electrophysiological events in the heart differ from those observed following anoxia. In another test he observed that lamprey killed with TFM did not have the methemoglobin which is present with anoxia. Smith and King (1969) found that oxygen consumption increased in lamprey exposed to TFM and Bayluscide®. Recently Dr. John Lech at the Medical College of Wisconsin, Milwaukee, Wisconsin, reported that the toxic action may be related to catecholamine metabolism. This theory is being tested by the use of the drug, Dibenzylene® (Fish Control Laboratories, 1971a).

RESIDUES

Federal regulations on the use of pesticides require that residue levels of TFM be determined in natural waters, bottom sediments, fish and other organisms exposed to the lampricide. Index streams were selected for observation of any long-term effects and toxic residues which might build up in the stream fauna (Erkila, 1964). Analytical procedures for

separating, concentrating, and detecting these residues had to be developed. The first method studied involved the adsorption on activated carbon of TFM residues from natural waters. The water quality, temperature, and site of sample were believed to have some effect on the quantitative measurement. Thus, the whole procedure needed more study and was finally considered inadequate to detect lampricide residues (Daniels et al., 1963 and 1965).

The next method made use of ion-exchange resin and solvent extraction of residues from natural waters, fish tissue, and bottom sediments (Daniels et al., 1965). Colorimetric methods were available to detect TFM in natural waters, but only concentrations of 0.1 ppm or greater could be measured. Therefore, ion-exchange resins were used to separate TFM residues. These compounds then could be recovered as concentrates by elution with selective solvent mixtures. TFM was removed from the whole fish by three extraction methods. At that time only the colored isomeric compounds, which absorb light at 395 nm, could be detected and measured (Daniels et al., 1965). These methods were not effective for detecting TFM in fish, bottom sediments, and water exposed to normal concentrations of the toxicant (Smith, 1966; Billy et al., 1965). By using an infrared recording spectrophotometer, various amounts of TFM residues were recovered from green sunfish exposed to extremely high concentrations of TFM. The value of this method is limited because only high concentrations of TFM can be detected (Smith, 1966).

Magadanz and Kempe (1968) observed that TFM disappeared in 2 weeks from natural waters that were in contact with bottom sediments. The rapid rate of removal is decreased if phenol is added to the bottom sediment. The residues in the bottom sediment apparently degrade, liberating the fluoride ion. With TFM in water alone, no color loss was observed.

In further studies on the removal of TFM by river muds, Sutton (1970) found that high temperature and organic content increase the rate of removal. Phenol stops the removal process by destroying the bacteria which degrade TFM. In a test to determine the toxicity of the degradation products of TFM, a concentration of 30 ppm of TFM was allowed to degrade 3 months. It was tested then against goldfish and lampreys, but none of them died.

In a related study Kempe (in review) reported on his attempts to determine whether TFM remains in the aquatic environment or is degraded to simpler compounds. Apparently, TFM degrades in bottom muds due to the action of bacteria, probably *Pseudomonas* spp. The products of this degradation, when tested against sea lamprey and goldfish, are not toxic. Only one-fourth of the fluorine added was recovered, according to other tests.

Lech (1971) has attempted to define metabolites of TFM in rats so that analytical methods may be developed for residues in organisms and the environment. The major metabolite appears to be 3-trifluoromethyl-4-aminophenol (RTFM). Both TFM and RTFM are excreted in the urine as polar derivatives, some of which appear to be glucuronides. Dr. Lech is being assisted by the Fish-Pesticide Research Laboratory, Columbia, Missouri, in the identification of TFM metabolites through mass spectrometry (Lennon, 1971).

In an effort to develop methods for detection of TFM in the ppb (parts per billion) range, the General Electric Company (1971) is investigating the analysis of TFM by luminescence spectrophotometry. If successful, the method will enable simple and rapid detection of small amounts of TFM in water, bottom sediments and field organisms. The Fish Control Laboratories are investigating the use of

gas chromatographic methods to detect and measure residues of TFM in fish tissue (Hunn, 1971). The method utilizing solvent extraction, acid-base partitioning, and gas chromatography, is sensitive to 0.005 ppm of TFM or less, and preliminary studies indicate excellent recovery (Fish Control Laboratories, 1971b).

APPLICATION METHODS

Successful application of TFM requires good equipment, proper formulations, and trained men using proven techniques. Many procedures were developed during the first three treatments of experimental streams in 1958. These included: 1) preliminary survey of lamprey distribution, of kinds and abundance of lampreys, fishes, and other fauna, and of potential treatment points; 2) pre-treatment analyses to determine the amount and exposure to TFM needed as influenced by discharge and velocity of water, and the chemical and physical properties of the water; 3) actual application techniques such as operating the controlled pumping system and measuring the concentration of TFM in the streams; and, 4) post-treatment surveys of dead fauna (Applegate *et al.*, 1961; Johnson, 1959 and 1961; Kanayama, 1963; Baldwin, 1964; Wadden, 1968; Schneider, 1969). Smith, Applegate, and Johnson (1960 and 1961) used a colorimetric method to detect the distribution and concentration of TFM as it traveled downstream. The intense yellow color of TFM in an alkaline solution was measured and analyzed.

Several years were required to develop adequate equipment for survey and treatment procedures. A light, portable shocker was developed to survey the abundance and distribution of ammocoetes in inaccessible areas (Braem and Ebel, 1961). An anchor dredge was modified by Thomas (1960) to sample the larvae populations at the mouths of rivers. Marking larval lamprey with cadmium sulfide and mercuric sulfide yielded population statistics (Smith and

McLain, 1962; Hansen and Stauffer, 1964). Electrical barriers have been installed at various streams to determine the reduction of lamprey populations by TFM treatments (Tibbles, 1965). Portable metering devices (gravity, fuel, and electric) were developed to add precise volumes of liquid TFM into streams (Anderson, 1962). A pour-portioner drum meter, for example, was adapted to improve application of small volumes of TFM into streams (Tibbles, Lamsa, and Johnson, 1970; Dustin, 1970). Mobile wet laboratories were developed to facilitate bioassays in the field (Howell and Marquette, 1962). A photoelectric amplifier was modified for use as a dye detector to determine water movement (Ebel, 1962). It provided a continuous record of the duration and intensity of the tracer dye in a stream (U. S. Bureau of Commercial Fisheries, 1961).

Various compounds that might augment the activity of TFM were tested with the intent of reducing the amount of TFM needed. The most successful of these was Bayluscide® (5, 2'-dichloro-4'-nitro-salicylanilide). Addition of 2 percent by weight of this compound to the toxicant mixture reduced the amount of TFM needed by 50 percent (Howell *et al.*, 1964; Johnson and Lamsa, 1964; Howell and King, 1966a). Unfortunately the mixture had several disadvantages that TFM alone did not have. It was barely soluble in water, clogged the feeder apparatus, and caused more fish kills when applied in lower Michigan streams by standard methods and in other areas from aircraft (Johnson and Lamsa, 1964; Smith, 1966 and 1967b; Lamsa, 1968; Smith and King, 1970).

Granular Bayluscide® and other nitro-salicylanilides have been tried alone as sea lamprey larvicides in deep water as bottom poisons, lamprey irritants, and survey tools, but many of these substances do not have a wide enough safety

margin for fish and invertebrates, are influenced strongly by water quality, or information on their residues is lacking (Jacob, 1966; Marking *et al.*, 1970; French and Swartz, 1968; Howell and King, 1966b; Starkey and Howell, 1966; Taborsky, 1970; Morman, 1969; King and Howell, 1970; Lennon *et al.*, 1970).

Another compound, toxaphene, was reconsidered for treatment of lakes and estuaries after being tested by the Canadians. Although a large number of ammocoetes were killed at 100 ppb and most fish populations recovered within 1 year, the use of toxaphene was discontinued by the U. S. Bureau of Commercial Fisheries and the Great Lakes Fishery Commission (Gaylord and Smith, 1966).

A registered fish toxicant, antimycin, may offer a possible solution to treating selectively such difficult areas as oxbows, estuaries, and bays off the mouths of rivers. Ayerst Laboratories, Inc., is experimenting with various formulations that would allow the toxicant to perform as desired (Lennon, 1971).

Because TFM is less toxic in hard waters than soft, a group of compounds was tested to find a chelating agent that would suppress the ionization of interfering divalent metal ion (Johnson, 1970). Although the results proved that divalent metal ions cause a decrease in activity of TFM, the amount of chelating agent needed to offset the decrease would be too expensive.

The experiences of the men in the field brought about recommendations and changes in field procedures. Johnson (1963 and 1964) reported details of bioassays performed from 1958 to 1962, and changed the design and interpretation of bioassays to suit management needs. Observations by control crews indicated a need for consideration of the following factors: 1) synchronization of serial applications of TFM in a stream through accurate measurement of stream

velocity and of changes in velocity; 2) improved efficiency of control in areas of difficult access by spot treatments, longer treatments, and multiple applications of TFM; and, 3) acclimatization of healthy bioassay animals in proper habitats (Johnson, 1959; Johnson and Tibbles, 1962).

The need for a multifaceted, flexible, evolving system for suppressing sea lamprey may bring about integrated controls that include both chemical and biological components (Hanson, 1970).

REGISTRATION

The U. S. Bureau of Commerical Fisheries obtained registration of TFM on August 21, 1964, for limited use as a sea lamprey larvicide. Use was restricted to trained operators and authorized personnel only. On May 13, 1970, the U. S. Department of the Interior was notified that the registration was to be cancelled by the Environmental Protection Agency on December 31, 1970, because no tolerances for TFM in fish and water has been established (Fish Control Laboratory, 1971). An application for extension until December 31, 1971, however, was granted to the Bureau of Sport Fisheries and Wildlife in order that additional data on methodology and toxicology may be obtained (Anonymous, 1970). An outline of the research needed on the chemical and physical properties, toxicity, efficacy, and residues was prepared, and assignments of contracts for the research were executed early in 1971 (Fish Control Laboratory, 1971). Hopefully, the research will be sufficient to re-register TFM as a badly needed lampricide.

SUMMARY

TFM is a halogenated mononitrophenol that was developed and registered as a selective toxicant for the sea lamprey. Its biological activity against the sea lamprey is affected only slightly by temperature, but it is affected by high alkalinity and

conductivity and by pH's below 7.0 and above 7.8. Generally, aquatic organisms are not affected adversely by stream treatments, except when concentrations have to be increased because conditions interfere with the chemical activity. TFM does not harm mammals at concentrations used in stream treatments. Various methods have been employed to detect TFM residues in water, organisms, and bottom sediments, but no method at present detects TFM in the ppb range. Methods of application and equipment have been improved through observations in the field and research. There are still many unanswered questions regarding TFM, but research in progress may answer them sufficiently for TFM to remain registered as a lampricide.

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APPENDIX A - TECHNICAL DATA ON TFM

Alternative names:

3-trifluormethyl-4-nitrophenol (Beilstein 1966)
 3-trifluoromethyl-4-nitrophenol (Smith, 1967)
 $\alpha\alpha\alpha$ -trifluoro-4-nitro-m-cresol (Frear, 1969)
 1, 3, 6-nitrotrifluorocresol (DeBrouwer, 1930)
 $\alpha\alpha\alpha$ -trifluoro-4-nitro-metacresol (Baker, 1962)
 2-nitro-5-hydroxybenzotrifluoride (Whalley, 1949)
 6-nitro-3-hydroxy-1-trifluormethyl-benzol (Beilstein, 1966)
 $\alpha\alpha\alpha$ -trifluor-6-nitro-3-hydroxy-toluol (Beilstein, 1966)
 Lampricide 2770 (Moffett, 1958a)
 Dowlap F40 (U. S. Bureau of Commercial Fisheries, 1958b)
 Dowlap [®] F (Frear, 1969)
 Eelicide - TFM (Jobin and Unrau, 1967)

Chemical names:

$\text{CF}_3\text{C}_6\text{H}_3(\text{NO}_2)\text{OH}$ (Rose and Rose, 1966)
 $\text{C}_7\text{H}_4\text{F}_3\text{NO}_3$ (Beilstein, 1966)

Formulations:

Crystalline solid, liquid (Applegate et al. 1961; Rose and Rose, 1966)

Primary use:

Selective toxicant for larvae of sea lamprey; Re-registration of lampricide in progress (Fish Control Laboratory, 1971).

Secondary uses:

Dye intermediate (Anonymous, 1959)
 Snail control (Jobin and Unrau, 1967)
 Housefly control with phosphoric ester of

TFM (Scherer, Stähler, and Frensch, 1957)

Seed protectants (Baker, 1962)
 Aquatic weed control (Josephs, 1961)

Toxicity to fish:

MAC_{25}^5 to 44 ppm (Applegate and King, 1962)

Toxicity to birds:

Not tested

Toxicity to mammals:

No acute effects in deer and dairy **cattle**; acute oral LD50 for rabbit is 0.16 g/kg, acute dermal LD50 at 1.6 g/kg (Applegate et al., 1961)
 Oral LD50 for rat is 0.5 to 1.0 g/kg of body weight (Frear, 1969)
 Pure TFM LD50 for rat is 30 mg/kg (Scherer, Frensch, and Stähler, 1964)
 50 percent of sodium salt of TFM oral LD50 for rat is 300 mg/kg (Scherer, Frensch, and Stähler, 1964)
 51 percent of TFM LD50 for rat is 200 mg/kg (Scherer, Frensch, and Stähler, 1964)

Safety hazards:

Extreme care needed in handling concentrated forms of toxicant; protective clothing, rubber gloves, and face masks recommended (Applegate et al., 1961)

Persistence in environment:

Non-persistent (Billy et al., 1965; Magadan and Kempe, 1968; Sutton, 1970)

APPENDIX B - COMMON AND TECHNICAL NAMES OF FISHES

The following fish classification was obtained by utilizing Bailey (1970).

<u>Common name</u>	<u>Technical name</u>	<u>Common name</u>	<u>Technical name</u>
LAMPREYS	PETROMYZONTIDAE	FRESHWATER	ICTALURIDAE
	<u>Ichthyomyzon spp</u>	CATFISHES	
	<u>Lampetra spp</u>	Bullheads	<u>Ictalurus spp</u>
American brook lamprey	<u>Lampetra lamottei</u>	Yellow bullhead	<u>Ictalurus natalis</u>
Sea lamprey	<u>Petromyzon marinus</u>	Channel catfishes	<u>Ictalurus punctatus</u>
		Stonecat	<u>Noturus flavus</u>
TROUTS	SALMONIDAE	TROUTPERCHES	PERCOPSIDAE
Coho salmon	<u>Oncorhynchus kisutch</u>	Trout-perch	<u>Percopsis omiscomaycus</u>
Chinook salmon	<u>Oncorhynchus tshawytscha</u>		
Rainbow trout	<u>Salmo gairdneri</u>	LIVEBEARERS	POECILIIDAE
Brown trout	<u>Salmo trutta</u>	Guppy	<u>Poecilia reticulata</u>
Brook trout	<u>Salvelinus fontinalis</u>		
MUDMINNOWS	UMBRIDAE	SUNFISHES	CENTRARCHIDAE
Central mudminnow	<u>Umbra limi</u>	Rock bass	<u>Ambloplites rupestris</u>
		Green sunfish	<u>Lepomis cyanellus</u>
PIKES	ESOCIDAE	Pumpkinseed	<u>Lepomis gibbosus</u>
Northern pike	<u>Esox lucius</u>	Bluegill	<u>Lepomis macrochirus</u>
MINNOWS AND CARPS	CYPRINIDAE	Smallmouth bass	<u>Micropterus dolomieu</u>
Goldfish	<u>Carassius auratus</u>	Largemouth bass	<u>Micropterus salmoides</u>
Carp	<u>Cyprinus carpio</u>		
Golden shiner	<u>Notemigonus crysoleucas</u>	PERCHES	PERCIDAE
Blacknose shiner	<u>Notropis heterolepis</u>	Yellow perch	<u>Perca flavescens</u>
Bluntnose minnow	<u>Pimephales notatus</u>	Logperch	<u>Percina caprodes</u>
Fathead minnow	<u>Pimephales promelas</u>	Walleye	<u>Stizostedion vitreum vitreum</u>
SUCKERS	CATOSTOMIDAE	SCULPINS	COTTIDAE
White sucker	<u>Catostomus commersoni</u>		

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INVESTIGATIONS IN FISH CONTROL

- 45. Residues of MS-222
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- 46. Methods of Estimating the Half-Life
of Biological Activity of Toxic Chemicals
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INVESTIGATIONS IN FISH CONTROL

45. Residues of MS-222 in Northern Pike, Muskellunge, and Walleye

By John L. Allen, Charles W. Lunning,
and Paul D. Harman

46. Methods of Estimating the Half-Life of Biological Activity of Toxic Chemicals in Water

By Leif L. Marking



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RESIDUES OF MS-222 IN NORTHERN PIKE, MUSKELLUNGE, AND WALLEYE

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ABSTRACT.--Residues of MS-222 (tricaine methanesulfonate) in muscle tissue of northern pike, muskellunge, and walleye following anesthesia were measured by a modified Bratton-Marshall colorimetric method and confirmed by thin-layer chromatography. The residues dissipate rapidly from the muscle when fish are withdrawn from the anesthetic and are near the background readings of the controls within 24 hours.

The U. S. Food and Drug Administration requires that certain disinfectants, antimicrobials, and anesthetics be cleared for their continued use on fish subject to human consumption. A part of the information necessary for clearance of MS-222 (tricaine methanesulfonate) as an anesthetic is its residues in treated fish. MS-222 has gained wide acceptance as an anesthetic for fish. The drug has been used to facilitate handling of fish during marking, spawn taking, and transporting operations.

The demand for popular species of fish such as northern pike (Esox lucius), muskellunge (Esox masquinongy), and walleye (Stizostedion vitreum vitreum) has become so great that they are extensively propagated in hatcheries. These fish are difficult to handle during such hatchery operations as spawning and marking unless they have been anesthetized. The release or use of fish treated with an anesthetic without prior knowledge of the persistence of the compound could result in consumption of an adulterated product.

Walker and Schoettger (1967a) measured MS-222 residues in four salmonids

following anesthesia, and Schoettger et al. (1967) measured MS-222 residues in muscle of channel catfish (Ictalurus punctatus).

This study was undertaken to measure residues of MS-222 in the muscle of northern pike, muskellunge, and walleye.

METHODS AND MATERIALS

Fish

The investigation of MS-222 residues in northern pike, muskellunge, and walleye was performed on fish treated with MS-222 in the springs of 1967 and 1968 (table 1). The fish were treated as outlined by Schoettger and Julin (1967) and Schoettger and Steucke (1970). Samples of muscle tissue collected by personnel of the Fish Control Laboratory in La Crosse, Wis., were individually wrapped, frozen, and shipped on dry ice to the Southeastern Fish Control Laboratory, Warm Springs, Ga., for analysis. Tissue samples of all species collected in 1967 were taken by removing a portion of muscle adjacent to the dorsal fin as described by Walker and Schoettger (1967b). Northern pike muscle tissue samples collected in 1968 were

Table 1.--Species and sources of fish

Common name	Scientific name	Average weight (grams)	Year	Site of treatment ^{1/}
Northern pike	<u>Esox lucius</u>	1,180	1967	NFH, Valley City, N. D.
		690	1967	NFH, Genoa, Wis.
		690	1967	FCL, La Crosse, Wis.
		1,178	1968	NFH, Valley City, N. D.
Muskellunge	<u>Esox masquinongy</u>	544	1967	NFH, New London, Minn.
		770	1967	NFH, Valley City, N. D.
Walleye	<u>Stizostedion vitreum vitreum</u>	1,286	1967	NFH, Genoa, Wis.
		1,286	1967	FCL, La Crosse, Wis.

^{1/} NFH = National Fish Hatchery; FCL = Fish Control Laboratory.

taken by filleting the fish and homogenizing the entire fillet before analysis.

Analysis

The samples from northern pike, muskellunge, and walleye treated in 1967 were analyzed by the modified Bratton-Marshall colorimetric method of Walker and Schoettger (1967b). In addition, at least one fish from each withdrawal interval was analyzed according to the thin-layer chromatographic method of Allen, Luhning, and Harman (1970), except that a developing solution of 2-percent methanol in benzene was used.

The samples of northern pike collected in 1968 were taken from homogenized fillets and were analyzed by the colorimetric method of Walker and Schoettger (1967a). Each sample was analyzed also by the ancillary thin-layer chromatographic (TLC) method of Allen, Luhning, and Harman (1970).

Although the colorimetric method provides the accuracy of a photometric measurement, background readings from naturally occurring primary aromatic amines prevent the detection of MS-222 residues less than background. The thin-layer chromatographic procedure was used to identify the residues of MS-222 and to

indicate when the residues had decreased to a concentration below the sensitivity of the procedure, which is approximately 0.2 ppm. Residues of less than 2.0 ppm are reported as a trace. The R_f of the sample spot was compared with the R_f of an MS-222 standard on the same plate to identify the residues as MS-222.

RESULTS

Northern pike

Residues of free MS-222 in northern pike treated in 1967 were measured by colorimetric analysis and averaged 8.4 ppm in the 0-hour withdrawal at 12° C to within the background readings of the controls at 24-hour withdrawal. The fish in the colder water accumulated higher residue concentrations at 0-hour than those in the warmer water. At all temperatures the 24-hour withdrawal samples were within the background readings of the controls. No residues of MS-222 could be detected even by the more selective thin-layer chromatographic analysis after 24 hours withdrawal (table 2). Residues of MS-222 were confirmed by thin-layer chromatography only in samples showing colorimetric readings higher than the control samples. One control sample showed a trace of MS-222 by thin-layer chromatography, but this sample may have been contaminated with MS-222 during

Table 2:--Residues of MS-222 including background aromatic amines in muscle tissue of northern pike at selected temperatures and withdrawal intervals following deep anesthesia with a 150 ppm solution of MS-222

Temperature and withdrawal interval	Site and year of treatment	Number of fish analyzed	Bratton-Marshall method-- residues in ppm				TLC method -- estimated free MS-222 residues in ppm <u>2/</u>
			Free MS-222		Free and acetylated MS-222		
			Mean	Range	Mean	Range	
8° C							
Control	Valley City NFH	1967 3 (1)	0.7	0.1- 1.8	2.0	1.5- 2.4	ND
0-hour	Do	1967 3 (1)	10.9	5.4-16.0	11.3	5.4-18.0	7.0
2-hour	Do	1967 3 (1)	5.3	2.4- 9.0	6.2	4.8- 8.4	4.0
24-hour	Do	1967 3 (1)	0.8	0.0- 1.2	1.3	0.8- 2.0	ND
12° C							
Control	Genoa NFH	1967 3 (1)	1.2	0.4- 2.8	2.6	2.0- 3.0	Trace
0-hour	Do	1967 3 (1)	8.4	3.2-17.0	9.1	5.4-15.0	6.7
2-hour	Do	1967 3 (1)	2.9	1.6- 4.6	5.7	4.0- 7.0	4.0
24-hour	Do	1967 3 (1)	0.8	0.5- 1.0	2.0	1.5- 2.4	ND
17° C							
Control	La Crosse FCL	1967 3 (3)	1.0	0.0- 2.4	2.1	1.5- 2.8	ND
0-hour	Do	1967 3 (1)	7.3	3.0-10.6	8.3	2.8-13.0	6.7
2-hour	Do	1967 3 (1)	0.7	0.0- 2.0	0.5	0.0- 1.0	ND
6-hour	Do	1967 3 (2)	0.3	0.0- 1.0	0.5	0.1- 1.0	ND
24-hour	Do	1967 2 (2)	1.1	0.0- 2.2	1.0	1.0- 1.0	ND
12° C							
Control	La Crosse FCL	1968 3 (3)	0.1	0.0- 0.2	1.4	1.0- 1.6	ND
0-hour	Do	1968 3 (3)	19.5	12.0-30.0	22.0	16.8-28.0	14.0
6-hour	Do	1968 3 (3)	0.1	0.0- 0.2	0.7	0.2- 1.0	ND
24-hour	Do	1968 3 (3)	0.0	0.0- 0.0	0.7	0.6- 1.0	ND
17° C							
Control	La Crosse FCL	1968 3 (3)	0.1	0.0- 0.2	1.8	1.4- 2.4	ND
0-hour	Do	1968 3 (3)	9.4	6.0-12.0	12.2	9.0-15.0	9.4
6-hour	Do	1968 3 (3)	1.6	1.0- 2.4	2.0	1.6- 2.8	Trace
24-hour	Do	1968 3 (3)	0.7	0.2- 1.0	1.1	1.0- 1.4	Trace <u>3/</u>

1/ Number in parentheses indicates the number of fish analyzed by the TLC method.

2/ ND = None detected; Trace = less than 2.0 ppm.

3/ Two of the three fish in this group showed a trace of MS-222 residue, but no residue was detected in the third fish.

analysis. There was not enough sample remaining to repeat the analysis.

In 1968, northern pike were anesthetized to medullary collapse with 150 ppm of MS-222 at 12° and 17° C at the Fish Control Laboratory at La Crosse, Wis. Residues of free MS-222 measured by colorimetric analysis averaged 19.5 ppm at 0-hour withdrawal to within the background readings of the controls at 6-hour withdrawal in fish treated at 12° C. Thin-layer chromatographic analysis of each fish from each withdrawal interval confirmed the presence of MS-222 residues only at the 0-hour withdrawal interval (table 2). At 17° C, residues of free MS-222 determined by colorimetric analysis averaged 9.4 ppm at 0-hour to trace after 24 hours withdrawal from the anesthetic. Thin-layer chromatography confirmed the presence of MS-222 residues in all the fish treated at this temperature with the exception of one fish from the 24-hour withdrawal interval.

Muskellunge

Residues of free MS-222 in muskellunge anesthetized to medullary collapse with 150 ppm of MS-222 at 10° C at the New London National Fish Hatchery in Minnesota averaged 7.9 ppm at 0-hour withdrawal, decreasing to background during 24 hours of recovery in fresh water (table 3). Thin-layer chromatographic analysis of two fish from each withdrawal period confirmed the presence of MS-222 residues at the 0-hour and 2-hour withdrawals. No residues were detected at the 24-hour withdrawals by TLC.

Walleye

In walleye treated at 17° C with 100 ppm of MS-222, residues of free MS-222 averaged 2.4 ppm at 0-hour withdrawal, and had decreased to within the background readings of the control samples during 24 hours of recovery in fresh water (table 4). Thin-layer chromatography of one fish

from each withdrawal interval confirmed the presence of MS-222 residues in fish from all withdrawal intervals except those at 24 hours. The 6-hour withdrawal sample showed only a trace of MS-222 residue remaining.

Walleye exposed to 120 ppm of MS-222 at 10° C at the Genoa National Fish Hatchery in Wisconsin showed residues of free MS-222 at 0-hour that averaged 4.3 ppm and decreased to within the background levels of the controls after 24 hours in fresh water (table 4). Thin-layer chromatography of one fish from each withdrawal interval confirmed the presence of MS-222 at each withdrawal interval except at 24 hours.

DISCUSSION

MS-222 residues remaining after 24 hours withdrawal from the drug were shown to vary little from the background amines of the controls as detected by colorimetric analysis. The colorimetric analysis for total free plus acetylated aromatic amines generally showed only slightly higher concentrations than the analysis for free aromatic amines only. Certain samples showed slightly higher readings for free MS-222 than for both free and acetylated aromatic amines. It is possible that small amounts of some of these amines could have been lost during the digestion step.

The identification of the residues was made by comparing the R_f of the spot obtained from the sample to the R_f of the spot given by the MS-222 standard. The semi-quantitation of the residues by thin-layer chromatography was accomplished by comparing the size and intensity of the sample spot with a series of standard spots of known concentrations. The quantitative estimates made by thin-layer chromatography were in agreement with the results obtained by colorimetric analysis. This indicates that most of the aromatic amine above the background reading of the controls detected by the colorimetric method was MS-222.

Table 3:--Residues of MS-222 including background aromatic amines in muscle tissue of muskellunge at selected withdrawal intervals following deep anesthesia with a 150 ppm solution of MS-222 at 10° C

Withdrawal interval	Site of treatment	Bratton-Marshall method--residues in ppm						TLC method -- estimated free MS-222 residues in ppm (mean) 1/ 2/	
		Free MS-222		Free and acetylated MS-222					
		Mean	Range	Mean	Range				
Control	New London NFH	0.3	0.1- 1.0	1.2	1.0- 1.5	(3)	ND	(2)	
0-hour	Do	7.9	3.4-10.5	10.7	7.6-13.0	(3)	4.3	(2)	
2-hour	Do	0.8	0.2- 1.0	2.2	1.5- 2.8	(3)	Trace	(2)	
24-hour	Do	0.4	0.0- 1.0	2.0	1.0- 2.8	(3)	ND	(2)	

1/ Number of fish in parentheses.

2/ ND = None detected; Trace = less than 2.0 ppm.

Table 4:--Residues of MS-222 including background aromatic amines in muscle of walleye at selected concentrations, temperatures, and withdrawal intervals following deep anesthesia

Temperature, concentration, and withdrawal interval	Sites of treatment	Bratton-Marshall method-- residues in ppm						TLC method-- estimated free MS-222 residues in ppm <u>1/</u> <u>2/</u>	
		Free MS-222		Free and acetylated MS-222					
		Mean	Range	Mean	Range				
10° C, 120 ppm									
Control	Genoa NFH	1.0	0.6-2.0	1.1	0.3-2.0	(3)	ND	(1)	
0-hour	Do	4.3	1.0-7.6	3.6	0.5-7.6	(3)	8.0	(1)	
2-hour	Do	1.8	0.4-4.0	1.0	1.0-1.0	(3)	Trace	(1)	
6-hour	Do	1.6	0.4-4.0	0.4	0.0-0.8	(3)	Trace	(1)	
24-hour	Do	0.4	0.0-1.0	1.0	0.5-2.2	(3)	ND	(1)	
17° C, 100 ppm									
Control	La Crosse FCL	1.7	0.4-2.8	2.3	2.0-2.8	(3)	ND	(1)	
0-hour	Do	2.4	0.4-3.6	2.5	1.0-3.6	(3)	4.0	(1)	
2-hour	Do	2.8	1.0-5.0	3.3	2.0-5.4	(3)	Trace	(1)	
6-hour	Do	2.0	1.4-2.8	2.5	2.0-2.8	(3)	Trace	(1)	
24-hour	Do	1.3	1.0-2.0	2.3	1.2-3.4	(3)	ND	(1)	

1/ Number of fish in parentheses.

2/ ND = None detected; Trace = less than 2.0 ppm.

With the exception of two samples of northern pike (table 2), no MS-222 residues were detected by thin-layer chromatography in fish withdrawn from the drug for 24 hours. Two of the three northern pike treated at 17° C and withdrawn from the anesthetic for 24 hours in 1968 showed

a trace of MS-222 by thin-layer chromatography, but no residue was detected in the third sample. The lower limit of sensitivity of the thin-layer chromatographic procedure is 0.2 µg of MS-222, but quantitation generally is not possible in samples spiked at less than 2.0 ppm.

Thus a trace of MS-222 as detected by TLC indicates the presence of MS-222 at concentrations of 2.0 ppm or less. The concentration of free MS-222 including background amines for the two 24-hour withdrawal samples treated at 17° C in 1968 was 1.0 ppm by colorimetric analysis. Both the thin-layer chromatographic and colorimetric method showed that these two samples contained less than 2.0 ppm of MS-222 residue.

Walleye were treated at a lower concentration of MS-222 and had the lowest concentration of MS-222 residues at 0-hour withdrawal. Northern pike had the highest concentration of MS-222 residues at 0-hour withdrawal. At the end of 24 hours, however, there were no significant differences in residue concentrations in any of the samples.

SUMMARY

Residues of MS-222 in muscle of northern pike, muskellunge, and walleye following anesthesia at selected temperatures were measured by the modified Bratton-Marshall colorimetric method (Walker and Schoettger, 1967b) and confirmed by thin-layer chromatography (Allen, Luhning, and Harman, 1970).

The mean concentration of MS-222 residues at the 0-hour withdrawal interval ranged from 2.4 to 19.5 ppm. The level of MS-222 residues at 0-hour withdrawal was highest in northern pike and lowest in walleye. After 24 hours withdrawal from the anesthetic, residues were detected in only 2 of the 12 northern pike analyzed by thin-layer chromatography, and these two samples were shown to contain less than 2.0 ppm.

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INVESTIGATIONS IN FISH CONTROL

46. Methods of Estimating the Half-Life of Biological Activity of Toxic Chemicals in Water

By Leif L. Marking



United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife
Washington, D.C. • July 1972

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METHODS OF ESTIMATING THE HALF-LIFE OF BIOLOGICAL ACTIVITY OF TOXIC CHEMICALS IN WATER

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ABSTRACT. -- In the absence of analytical methods, the half-life of biological activity of a chemical (the time required to decrease its toxic activity by one-half) can be estimated by bioassays. The methods presented require the determination of LC50 values (concentration producing 50% mortality) for organisms in aged solutions containing unknown residual concentrations, and concurrent tests of solutions containing known concentrations. The half-life of biological activity is determined by plotting (1) the percent concentrations remaining in aged solutions or (2) the deactivation indices against aging time on cyclic semilogarithmic graph paper. By the first method, a 5-day half-life for antimycin, a fish toxicant, was determined using resistant channel catfish (Ictalurus punctatus) and goldfish (Carassius auratus) and for sensitive green sunfish (Lepomis cyanellus) and rainbow trout (Salmo gairdneri) in soft water at pH 7.5. The second method, using only rainbow trout, also estimated a 5-day half-life for antimycin in soft water at pH 7.5. These methods could be used also to determine the half-life of antimycin at different pH's, temperatures, and light intensities, the factors thought to influence the toxicant's efficacy. The methods are not limited to fish and fish toxicants.

The biological activity of a toxicant is the killing power, which decreases as the toxicant is deactivated biologically and chemically with time. The half-life of a toxicant's biological activity is the time necessary for that activity to be reduced by one-half. Regulatory agencies now require data on the persistence of toxicants in the environment, and the half-life or rate of disappearance is a good measure of persistence.

The half-life of most fish toxicants in water is influenced by pH, temperature, sunlight, metabolism by aquatic organisms, and other environmental variables.

Analytical methods to detect and measure very small concentrations of some fish toxicants are unavailable. For ex-

ample, antimycin, a fish toxicant, kills rainbow trout at 30 parts per trillion (Berger et al., 1969), but analytical methods aren't available to quantitate this concentration. Walker et al. (1964) observed that antimycin degrades rapidly in water, especially under alkaline conditions, and recommended further definition of the detoxification rate.

In lieu of inadequate analytical methods, I have outlined two laboratory bioassay methods, free of analytical instrumentation, to estimate the half-life of biological activity for toxicants used in water. The first method requires test animals that are differentially sensitive to a toxicant in order to quantitate the decreased concentrations resulting from degradation. The second method requires one test species

and greater than lethal concentrations of a chemical that are aged to levels commensurate with the tolerance of the selected species.

Mortalities in the bioassays with degrading concentrations are compared with those in concurrent reference tests on the same organism using known concentrations of the toxicant. The experiments with antimycin included sensitive and resistant species of fish, but other aquatic organisms such as mollusks, aquatic insects, and plants may be used in the bioassays for the half-life determination. The intra-species tolerance differences are of little consequence in the procedure since a reference bioassay is conducted concurrently with the degrading solutions.

The half-life of biological activity of a toxicant can be determined for various environmental parameters such as pH and temperature. Such information would be invaluable to fishery biologists in selecting minimum lethal concentrations of fish toxicants or aquatic herbicides for target species and in avoiding hazards to non-target species. Field biologists using antimycin have reported incomplete kills of fish that probably resulted more from rapid deactivation of the antibiotic in water of a particular quality than from applying a nontoxic concentration. For example, 1 ppb of antimycin may kill all of the target fish in water of pH 8, but fail to kill any in water of pH 9. Knowledge of chemical persistence of toxicants in the aquatic environment is essential for efficacious, safe, and economical control of target organisms.

METHODS

The fish toxicant antimycin was chosen as an example to determine the half-life of biological activity according to the methods described. Previous reports indicate that the toxicity of antimycin is influenced by chemical and biological properties of the

water, especially those controlling pH, but no one has been able to quantify the rate of deactivation. The static bioassay routine and maintenance of test fish were essentially those described by Lennon and Walker (1964) and Hunn et al. (1968). All the tests were conducted in soft water at pH 7.5 and 12° C.

Method A

The concentration of biologically active chemicals remaining in solutions permitted to age for a selected period of time can be estimated by introducing fish or other susceptible aquatic organisms for bioassay. More resistant species are exposed to lethal solutions of toxicants that have been aged slightly, whereas more sensitive species are exposed to older solutions that have become deactivated to lower concentrations. Concurrently with tests in aged media, identical organisms must be employed in bioassays of fresh solutions under identical test conditions and exposure periods at concentrations expected to produce mortality. The mortality data from the tests in aged and fresh solutions are analyzed to determine the statistical LC50 (concentration producing 50 percent mortality) according to the method of Litchfield and Wilcoxon (1949). The LC50's are calculated for both tests on the basis of applied concentrations even though the concentration has decreased in the aging tests.

To determine the approximate concentration remaining in the aged bioassays, a formula was developed using hypothetical data for ideal disappearance of biological activity presented in table 1. The hypothetical data are characteristic for a toxicant with a half-life of 1 day. LC50's for all of the tests in aged solutions (C_1) are identical at 50 ppb in this hypothetical case. In actuality, the statistical LC50 would not be consistent for each test, but I have kept it constant for simplicity. The actual concentrations remaining in the aged

solutions are unknown after aging begins, yet the LC50's are calculated from the concentrations originally applied to the test water. In reference tests with fresh solutions (C_2), the concentrations required decrease proportionately with the deactivation of aged solutions, and therefore more sensitive species are required to conform to these lower concentrations. The percent concentration remaining is calculated according to the following equation:

$$\text{Percent concentration remaining} = 100 - \left[\frac{(C_1 - C_2)}{C_1} \right] 100$$

Where C_1 = LC50 of aged solutions

C_2 = LC50 of fresh solutions

This computation is repeated for each aging time interval.

A plot of the percent concentration remaining and aging time (table 1) on semi-logarithmic graph paper reveals a typical first-order decay curve with a half-life of 1 day. The unit of days was chosen for the hypothetical situation, but hours or weeks are appropriate when toxicants are deactivated slower or faster.

Example

Four series of uniform concentrations of antimycin were set up and permitted to age prior to introducing the test species. Each series included concentrations of 20, 40, 60, 80, and 100 ppb of antimycin and a control with no toxicant. After 7 days of aging, 10 channel catfish were introduced into each concentration and the control vessel of a single series. On the same day, antimycin was added to five vessels containing 10 channel catfish each at concentrations ranging from 1 to 40 ppb. The 96-hour LC50 of the 7-day-old solutions was calculated to be 47.300 (C_1), and that value for the fresh solutions is 20.400 ppb

(C_2). By substituting the values of C_1 and C_2 into the formula, the percent concentration remaining after 7 days is calculated to be 43.13 (table 2).

Goldfish were added to the second series of antimycin concentrations of 20, 40, 60, 80, and 100 ppb after the solutions had aged for 30 days. Concurrently, goldfish were bioassayed in fresh solutions of antimycin, and the 96-hour LC50's were computed for both tests. The resulting percent concentration remaining after 30 days is 1.10 (table 2).

Green sunfish were added to another series of aged solutions after 35 days of deactivation, and rainbow trout after 44 days of deactivation. The percent concentration remaining was computed for both species (table 2).

The reference LC50's (C_1) show different sensitivity among the four species, and the 96-hour LC50's range from 20.400 ppb for the more resistant catfish to 0.049 ppb for the more sensitive trout. The LC50's are characteristic for these species in this water quality, but change in tests using different water.

The percent concentration remaining and aging time from table 2 are plotted on cyclic semilogarithmic graph paper (fig. 1). The curve approximates a first-order decay curve and describes the rate of disappearance for antimycin in soft water of pH 7.5. The percent concentration remaining is 50 after 5 days and 25 after 10 days; therefore the half-life of biological activity is 5 days.

Method B

The half-life of biological activity can be estimated by using only one species of test organism. The procedure requires aged solutions of toxicant, but stronger solutions are established and allowed to age for longer periods, such as 2, 4, 8,

Table 1:--Percent of concentrations remaining as calculated from hypothetical data on aged and fresh solutions of toxicant

Aging time for C ₁ (days)	LC50 1/ (ppb) of		Percent reduction of LC50	Percent concentration remaining
	Aged solutions (C ₁)	Fresh solutions (C ₂)		
0	50.000	50.000	0.00	100.00
1	50.000	25.000	50.00	50.00
2	50.000	12.500	75.00	25.00
3	50.000	6.250	87.50	12.50
4	50.000	3.125	93.75	6.25
5	50.000	1.563	96.87	3.13
6	50.000	0.782	98.43	1.57
7	50.000	0.391	99.21	0.79
8	50.000	0.196	99.60	0.40
9	50.000	0.098	99.80	0.20
10	50.000	0.049	99.90	0.10

1/ Calculated from original concentrations of aged and fresh solutions bioassayed concurrently.

Table 2:--Toxicity of antimycin to fish in aged and fresh solutions at pH 7.5 and 12° C.

Species	Age in days of aged test solutions	96-hour LC50 (ppb) of		Percent concentration remaining
		aged (C ₁) solutions	fresh (C ₂) solutions	
Channel catfish (<u>Ictalurus punctatus</u>)	7	47.300	20.400	43.13
Goldfish (<u>Carassius auratus</u>)	30	60.500	0.650	1.10
Green sunfish (<u>Lepomis cyaneus</u>)	35	52.000	0.308	0.59
Rainbow trout (<u>Salmo gairdneri</u>)	44	34.400	0.049	0.20

16, 32, or more days. As the solutions deactivate to a level of toxicity commensurate with the tolerance of the test organism, they are bioassayed against the

test species. Concurrently with the bioassay of aged solutions, fresh solutions are bioassayed against individuals of the same species to provide reference data.

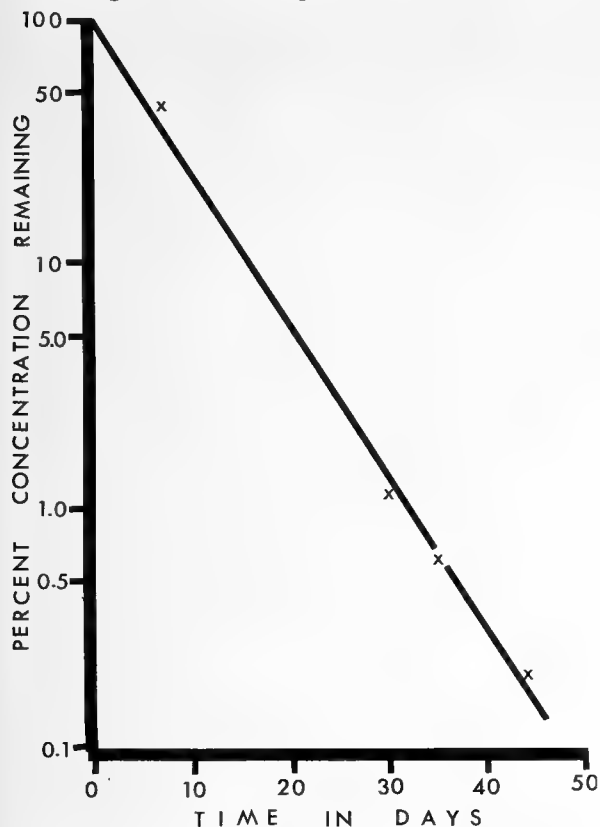


Figure 1:--The inactivation of antimycin at pH 7.5 and 12° C by method A.

The results obtained by bioassaying aged and fresh solutions against a single species are used to compute a deactivation index. The deactivation index is derived by dividing the LC50 of an aged solution by the LC50 of a fresh reference solution. The index has a value greater than 1 if the chemical deactivates at all. A value of 2 indicates that the concentration of an aged solution has diminished by one-half. The aging time required for a deactivation index of 2, calculated from the LC50's, is equivalent to the half-life of biological activity of the toxicant.

The deactivation index invariably does not equal exactly 2 for any particular test. Therefore, the deactivation index is computed for each aging period, and the results are plotted on semilogarithmic coordinates. The slope of the curve

influences the half-life value and is derived from a line fitting the plotted deactivation indices.

Example

Rainbow trout were selected to assess this alternate method for half-life of biological activity. Five series of different concentrations of antimycin were prepared on different days during a 16-day period. The first series of solutions contained higher concentrations than subsequent series because it would be deactivating for 16 days prior to introducing the fish. The next series aged for 8 days, and the initial concentrations were lower than in the first series. Another series of concentrations was aged 4 days, and another for 2 days prior to introducing the trout. All of the aged solutions were bioassayed on the same day. Concurrently, a reference bioassay with rainbow trout was conducted using known concentrations in fresh solutions. The 96-hour LC50's for solutions aged for different time periods were used to compute the deactivation indices (table 3).

The deactivation indices range from 1.25 to 7.5 and signify the deactivation occurring over the test period. They are plotted against aging time on semilogarithmic graph paper (fig. 2). From this graph, the half-life of biological activity for antimycin in soft water at pH 7.5 and 12° C is about 5 days. This value agrees with that determined by method A.

DISCUSSION AND CONCLUSIONS

The model (table 1) conveniently presents data for each 1-day interval of the aging process. Quite frequently, as in the example of antimycin, species are unavailable for consistent 1-day increments and consequently the aging times before introducing the species do not coincide with the half-lives. This does not prevent the half-life determination, however, since the

Table 3:--Toxicity of antimycin (ppb) to rainbow trout in aged (C_1) and fresh (C_2) solutions in 96-hour tests at pH 7.5 and 12° C.

Aged solutions		Fresh solutions LC50 (C_2)	Deactivation index
Age in days	LC50 (C_1)		
2	0.0498	0.040	1.25
4	0.0787	0.040	1.97
8	0.130	0.040	3.25
16	0.300	0.040	7.50

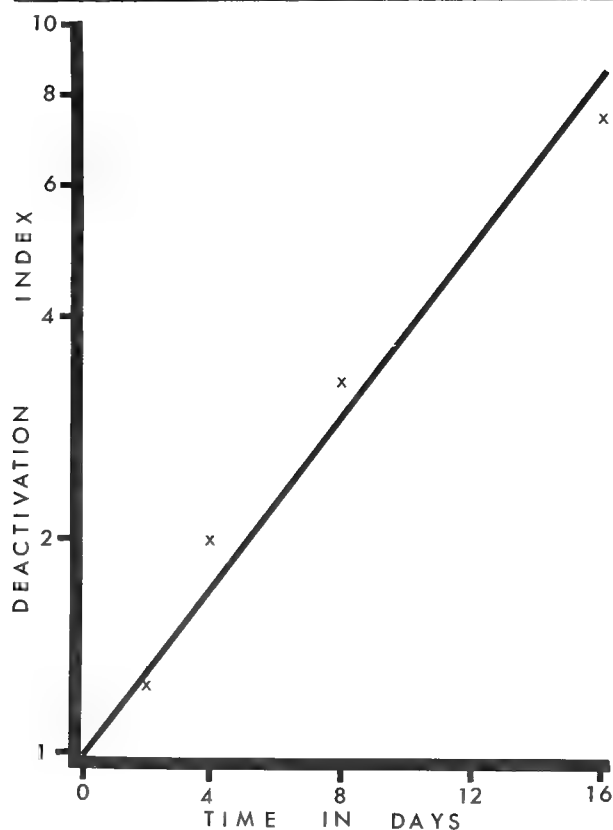


Figure 2:--The inactivation of antimycin at pH 7.5 and 12° C by method B..

percent reduction can be plotted at any time a species can be found which is sensitive to the aged solutions.

In field applications, target organisms must be exposed to a minimum lethal concentration or greater for a certain period

of time for successful eradications. Knowledge of the half-life of biological activity permits selection of concentrations which will retain sufficient toxicity throughout an exposure period to kill the target organism. In addition, the concentration remaining in the treated water can be calculated for any reasonable length of time. For instance, highly sensitive species may be introduced into waters in which highly resistant species were eradicated. The time required for the toxicant to become sublethal to the sensitive species can be extrapolated from the half-life curve.

In the event that a chemical detoxifies too slowly and bioassay space and equipment cannot be tied up for long periods, the procedure for method A may be divided into segments which can be accomplished concurrently. For example, resistant species may be tested in aged (C_1) and fresh (C_2) solutions while at the same time sensitive species may be tested in lower concentrations of aged (C_1) and fresh (C_2) solutions. The percent reduction in concentrations for each time segment is plotted on a single graph and the half-life is obtained by averaging the rates for individual species. This practice reduces the time that single solutions must age, thereby reducing problems caused by evaporation and decreasing the chance of contamination in the test solutions. It also detects changes in rates of detoxification for different concentrations of toxicants.

A difficulty sometimes arises in determining the proper time to bioassay the aged solutions, especially without prior knowledge of the toxicant's persistence. After the initial test in method A, however, the data can be plotted using the single percent concentration remaining and assuming 100 percent biological activity at the beginning of the test. The slope of the curve may be influenced in subsequent tests, but through a quick half-life approximation the activity can be predicted for longer aging periods and more sensitive species. Following the tests of more sensitive species, the curve is redrawn for best fit to all of the calculated values.

Method A is limited by the number of differentially sensitive organisms available. In fact, to more accurately derive the half-life by this method, three or more values are desirable to establish the slope of the curve. Several of these values may be obtained using one species, although more than one species should be used in deriving the half-life curve. The model presents a reduction in toxicity by a factor of 1,000 using 10 different aging periods, but experimental chemicals frequently are not that differentially toxic nor are that many species available.

Advantages of method B are that only one species is required, fewer reference tests are necessary, and the total time and facility requirements are less. Both methods are estimates because the values derived are taken from a curve representing statistical calculations on the tolerance of biological organisms. The plotted values are experimental and do not necessarily occur on the curve. In method A the variation is among different species, whereas in method B the variation is among different tests of the same species. For better results, several species could be tested in method B and values could be averaged.

Neither method is intended to account for absorption and adsorption of test chemicals to different kinds of bioassay vessels or for complexing to organic matter found in different waters. Also, they do not differentiate between chemical degradation and biological inactivation. The methods estimate the available total activity of a chemical and its degradation products under defined test media conditions.

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INVESTIGATIONS IN FISH CONTROL

- 47. Preparation and Properties of Quinaldine Sulfate,
an Improved Fish Anesthetic**
- 48. Toxicity of Quinaldine Sulfate to Fish**
- 49. The Efficacy of Quinaldine Sulfate as an
Anesthetic for Freshwater Fish**
- 50. Residue of Quinaldine in Ten Species of Fish
Following Anesthesia with Quinaldine Sulfate**



United States Department of the Interior
Fish and Wildlife Service
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FOREWORD

An anesthetic used on food and game fishes must bear a label registered by the U.S. Food and Drug Administration. The requirements for registration include data on the toxicity of the drug to exposed fish; on the efficacy as an anesthetic to selected fishes of several life stages in waters of different pH, hardness, and temperature; and on the residues of drug present in edible fish tissues following exposure. The following papers are concerned with results of registration-oriented research on toxicity, efficacy, and residues of quinaldine sulfate (QdSO_4) when employed as an anesthetic for selected species of coldwater and warmwater fish. The data will be used to support a petition for registration of the anesthetic.

Chemists at the Fish Control Laboratories developed quinaldine sulfate as an improved formulation of quinaldine, a coal tar derivative used extensively in the manufacture of dyes, pharmaceuticals, and fine organic chemicals. Quinaldine came into use as a fish anesthetic in 1958 and is preferred in some fish culture and fishery management operations because of its low order of toxicity to fish in long exposures. Although effective and economical, the 95-percent quinaldine used is an oily liquid that is insoluble in water and possesses a strong, disagreeable odor. In addition to these disadvantages, the 5 percent of other quinones present would have to be identified and studied in detail before an attempt was made to obtain registration of quinaldine as a fish anesthetic.

In contrast, quinaldine sulfate is a pure, crystalline formulation that is water soluble, has little odor, is easy to handle, and is effective as a fish anesthetic. Its purity would be an advantage in seeking registration. Thus, we chose to do registration-oriented research on quinaldine sulfate rather than on quinaldine.

Robert E. Lennon, Director
Fish Control Laboratories
June 2, 1972

47. Preparation and Properties of Quinaldine Sulfate, an Improved Fish Anesthetic

By John L. Allen and Joe B. Sills



United States Department of the Interior, Rogers C. B. Morton, *Secretary*
Nathaniel P. Reed, *Assistant Secretary for*
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PREPARATION AND PROPERTIES OF QUINALDINE SULFATE, AN IMPROVED FISH ANESTHETIC

By John L. Allen and Joe B. Sills
Fish Control Laboratories, La Crosse, Wisconsin
and Warm Springs, Georgia

ABSTRACT.--The preparation of quinaldine sulfate (QdSO_4) from practical grade quinaldine and sulfuric acid yields a crystalline product which is water soluble and easily purified by recrystallization. The product conforms with the empirical formula $\text{C}_{10}\text{H}_{11}\text{NSO}_4$ and assays 99.4 percent pure by titration. The crystalline material has little odor and is water soluble; thus, it is less objectionable and much easier to handle than quinaldine for anesthetizing fish.

INTRODUCTION

Quinaldine (2-methylquinoline) was suggested as an anesthetic for fish by Muench (1958) and has been used safely and effectively to facilitate general handling of fishes over a wide variety of conditions. This chemical presumably enters a fish's body by absorption across the gill membranes, but its specific physiological mode of action has not been determined. At a concentration of 10 mg/l, quinaldine has been shown by Clark and Granath (1968) to depress the frequency of electrical discharge of the gymnotid eel (*Sternarchus albifrons*). Trams and Brown (1970) using brain homogenates from nurse sharks (*Ginglymostoma cirratum*) and cow-nosed rays (*Rhinoptera bonasus*) have shown that 10^{-3} M quinaldine produced a 50-percent inhibition of acetylcholinesterase and butyrylcholinesterase. However, Trams and Brown do not think this is the mode of action of quinaldine.

Practical grade quinaldine is an economical and effective tool; however, it is an oily liquid not readily soluble in water, and has a strong, disagreeable odor. Jodlbauer and Salvendi (1905) noted that quinoline and the sulfuric acid salt of quinoline both exhibited anesthetic properties with fish. Sills and Harman (1970) showed quinaldine sulfate possesses the same desirable anesthetic properties as quinaldine. The salt is a

crystalline form of the anesthetic with none of the unfavorable characteristics of the liquid, and is a product of high purity. The objectives of this paper are to present the preparation of the quinaldine salt and its chemical characterization as an improved formulation for use as an anesthetic by fishery workers.

METHODS AND MATERIALS

Since commercial sources of quinaldine sulfate could not be located, the quinaldine sulfate was prepared from Eastman Kodak, 95-percent quinaldine. Initially, two small batches of quinaldine sulfate were prepared by reacting 20 ml of quinaldine with 7.0 ml of concentrated sulfuric acid in the presence of a hydrocarbon solvent. Later, four batches of quinaldine sulfate (2-methylquinoline sulfate) were prepared using the following batch formula:

quinaldine, 500 grams or 3.49 moles
sulfuric acid, 190 ml or 3.42 moles
iso-octane, 500 ml.

The quinaldine and iso-octane were placed in a 4,000-ml reaction flask and were mixed thoroughly with a mechanical stirrer. The reaction mixture was placed in an ice bath, and concentrated sulfuric acid was added slowly while the

mixture was stirred vigorously. The rate of sulfuric acid addition was adjusted to keep the temperature of the mixture below the boiling point of iso-octane (99.3° C.). The resultant mixture was filtered using vacuum, and the filter cake was washed with approximately 250 ml of benzene. The product was recrystallized from methanol. The recrystallization yielded approximately 566 grams of a bright red crystalline material (68.5-percent yield).

RESULTS

The quinaldine sulfate was tested for purity and for structural changes as a result of the reaction of sulfuric acid with quinaldine. The recrystallized quinaldine sulfate was soluble in water and alcohol and insoluble in benzene and ether (table 1.). The melting range of the material was 211°-214° C. Stecher et al. (1968) list the melting range of quinaldine sulfate as 211°-213° C. When titrated to a phenolphthalein end point with 0.1 N sodium hydroxide, the product was determined to be 99.4 percent $C_{10}H_{11}NSO_4$. The infrared spectra of practical grade quinaldine

and the free base of quinaldine sulfate were determined in carbon tetrachloride (from 4,000 to 1,300 cm^{-1}) and in carbon disulfide (from 1,300 to 400 cm^{-1}). The spectrum of free base of quinaldine sulfate shows all the absorption bands present in the spectrum of practical grade quinaldine (fig. 1). The infrared spectrum of quinaldine sulfate in a KBr pellet also contains bands at 1,000 and 1,170 cm^{-1} indicating presence of sulfate and a broad band at 2,700 cm^{-1} which indicates an amine salt. The ultraviolet spectrum of the product in 0.1 N H_2SO_4 gives absorption maxima at 236 and 317 nm (fig. 2).

An aqueous solution of quinaldine sulfate was made alkaline and extracted with hexane to give a solution of the free base of quinaldine in hexane. The resulting hexane solution was injected into a gas chromatograph equipped with a 180 cm x 4 mm column packed with 5-percent carbowax 20 M on gas chrom Q 80/100 mesh at 130° C. and a flow rate of 60 ml/minute. A single peak with the same retention time as quinaldine eluted after the solvent peak.

The product gives a positive test for sulfate using the USP XVII (1965) test for sulfates.

Two batches of quinaldine sulfate prepared by the above method were subjected to elemental analysis¹ with the following results.

	%C	%H	%N	%S	%O
Calculated	49.78	4.60	5.81	13.29	26.53
Sample 1	49.40	4.59	5.89	13.15	26.20
Sample 2	49.48	4.57	5.83	13.39	26.79

The elemental analysis indicates an empirical formula of $C_{10}H_{11}NSO_4$, which is the empirical

¹ The elemental analysis was done by Galbraith Laboratories, Inc., Knoxville, Tennessee.

TABLE 1.--The solubility of quinaldine sulfate in seven different solvents at 20° C.

Solvent	Solubility (g/100 ml)
water	104.05
methyl alcohol	7.44
ethyl alcohol	2.27
acetone	0.08
ethyl ether	¹ i
benzene	i
hexane	i

¹ Insoluble = less than 0.01 grams dissolves in 100 ml of solvent.

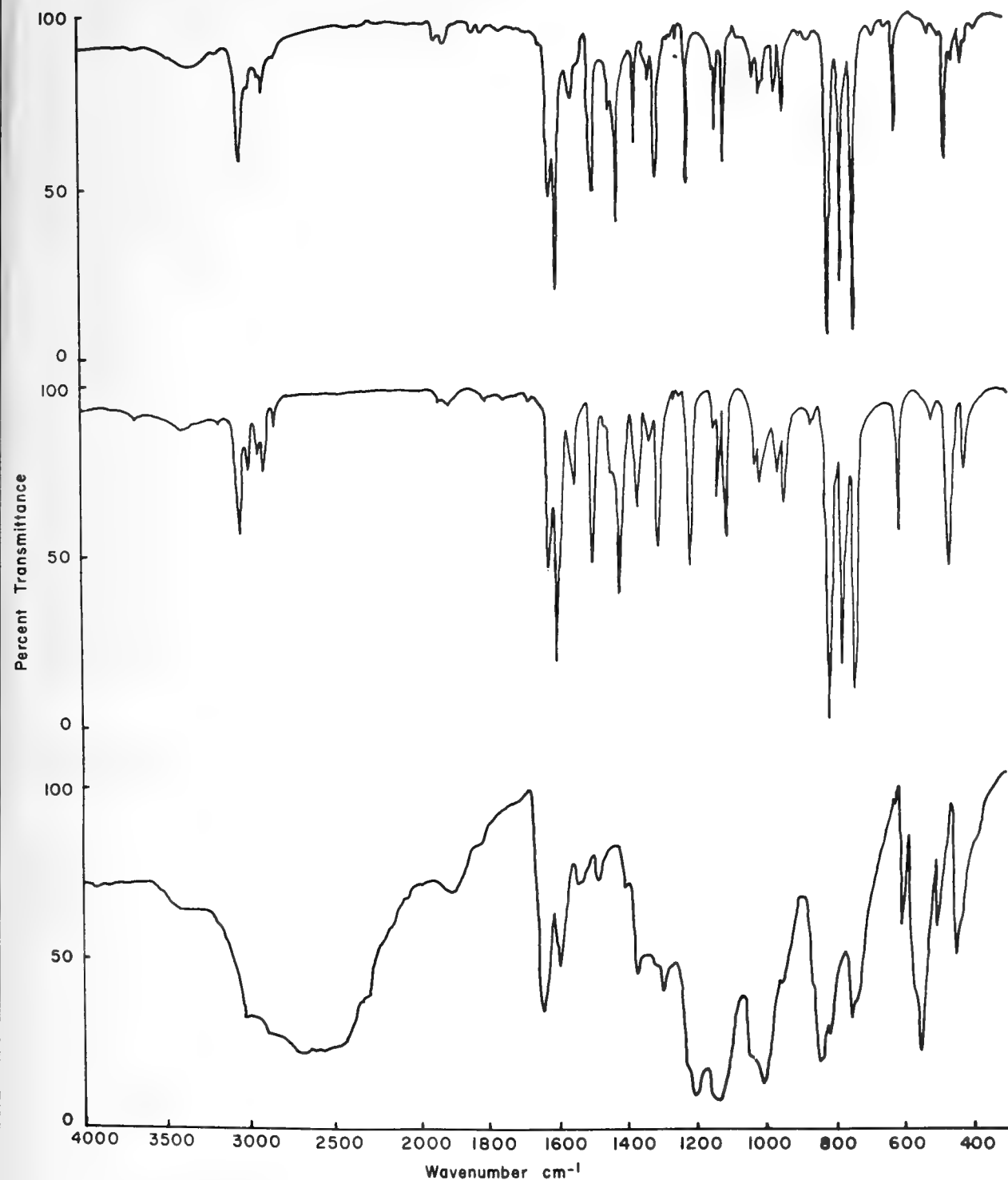


Figure 1.—Infrared spectra of Eastman Kodak practical grade quinaldine (top), and the free base of quinaldine sulfate (middle) in carbon tetrachloride and carbon disulfide, and quinaldine sulfate (bottom) in a KBr pellet.

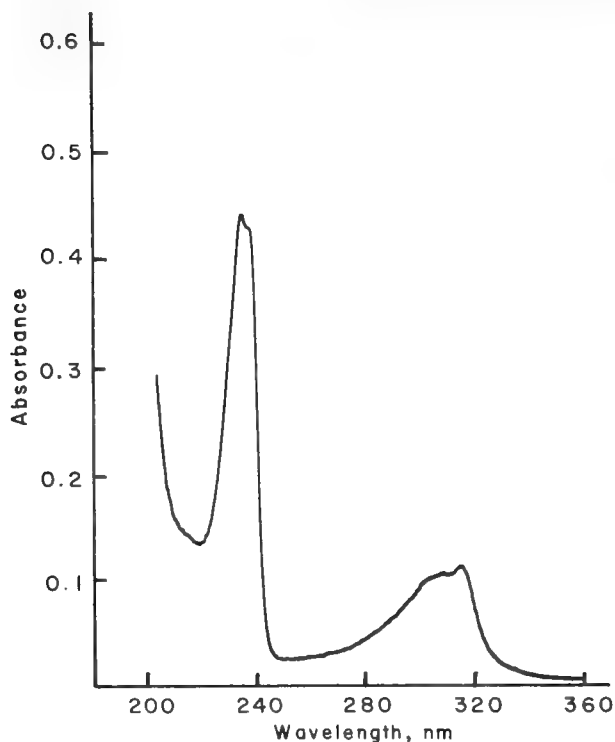
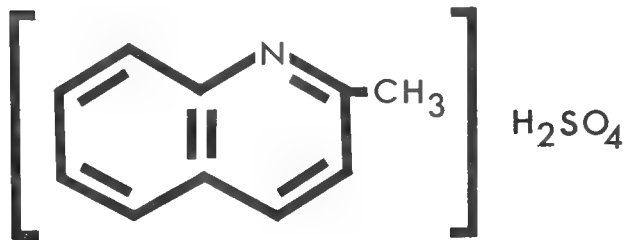


Figure 2.—Ultraviolet spectrum of quinaldine sulfate in 0.1 N sulfuric acid.

formula of quinaldine sulfate given by Stecher et al. (1968). The product can be represented by the semi-structural formula given below.



DISCUSSION

The quinaldine sulfate prepared during this study has been shown to be a product of high purity. The reaction of sulfuric acid with quinaldine using the conditions of preparation we used in this investigation did not alter the structure of the quinaldine molecule. The sulfate salt of the nitrogenous base (quinaldine) was recovered in good yield (68.5 percent).

Schoettger and Julin (1969) indicated that quinaldine is inactive as an anesthetic for fish at pH 6

or below. Sills and Allen (1971) correlated the efficacy of quinaldine at a given pH with the ionization constant of the molecule. They indicated the uptake of the chemical by fish is dependent on the amount of the quinaldine present in the water in the lipophilic, free base form. Quinaldine sulfate is an acid salt and will depress the pH of anesthetic solutions, particularly in soft water. The depression of pH can be overcome by buffering the anesthetic solutions.

CONCLUSIONS

1. Recrystallization of quinaldine sulfate prepared by the reaction of quinaldine with sulfuric acid gives a product of high purity.
2. Quinaldine sulfate is a water soluble crystalline material with little odor.
3. Since this salt is also an effective anesthetic, it is a more useful tool for fishery work than practical grade quinaldine.

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TOXICITY OF QUINALDINE SULFATE TO FISH

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ABSTRACT.--The acute toxicities of the candidate fish anesthetic, quinaldine sulfate (QdSO_4), were determined against selected species of cold-water and warmwater fishes. The LC_{50} 's (concentrations producing 50-percent mortality) were derived for 3-, 6-, 24-, and 96-hour exposures in bioassays with different temperatures, hardnesses, and pH's. The 96-hour LC_{50} 's of QdSO_4 ranged from 6.8 mg/l for largemouth bass to 72.5 mg/l for carp. In very soft water QdSO_4 solutions are acidic and considerably less toxic than in harder water. This lack of activity is attributed to a decrease in the pH of the test solution which thereby decreases the concentration of the active, un-ionized form of the molecule.

INTRODUCTION

Quinaldine sulfate (QdSO_4) is a candidate fish anesthetic (Allen and Sills, 1973). An early step in its development and possible registration as a fishery tool is definition of its toxicity to selected fishes in standard bioassays in waters with different hardnesses, pH's, and temperatures (Lennon, 1967). Observations also were made on the influence of quinaldine sulfate on bioassay media and on degradation of the compound.

METHODS AND MATERIALS

Static bioassays of QdSO_4 were conducted with 3- to 14-cm fish in 15-liter glass jars according to the methods of Lennon and Walker (1964).

Larger fish were exposed to the anesthetic in 45-liter polyethylene tanks. The fish were obtained from fish hatcheries, maintained under a fish culturist's care (Hunn, Schoettger, and Whealdon, 1968), acclimated according to standard bioassay procedures, and incinerated after death. Ten fish were exposed to each concentration of the anesthetic, and mortalities were recorded periodically during the first day and daily thereafter during the 96-hour tests.

Variations in test water were produced by adding different amounts of reconstituting salts to deionized water (table 1). The pH in various tests was adjusted and maintained with chemical buffers (table 2). Temperatures of 7⁰, 12⁰, and 17⁰ C. were controlled by water baths.

TABLE 1.--Quantities of salts and characteristics of reconstituted waters

Water type	Salts added (mg/l)				pH range	Total	
	NaHCO_3	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	MgSO_4	KCl		Hardness ²	Alkalinity ²
very soft	12	7.5	7.5	0.5	6.4-6.8	10-13	10-13
soft ¹	48	30	30	2.0	7.2-7.6	40-48	30-35
hard	192	120	120	8.0	7.6-8.0	160-180	110-120
very hard	384	240	240	16	8.0-8.4	280-320	225-245

¹ Standard reconstituted water used in routine bioassays.

² As mg/l CaCO_3 .

TABLE 2.--Buffer chemicals for adjusting the pH of standard reconstituted water at 12° C.

pH	Ml of solutions for 15 liters of water		
	1N NaOH	1M KH ₂ PO ₄	0.5M H ₃ BO ₃
6.0	1.3	80.0	--
6.5	10.0	30.0	--
7.0	19.0	30.0	--
7.5	--	--	--
8.0	19.0	20.0	--
8.5	12.0	11.5	--
9.0	8.8	--	30.0
9.5	11.0	--	20.0
10.0	16.0	--	18.0

The anesthetic was dissolved in water, and aliquots were added to the bioassays to obtain the desired concentrations.

The mortality data were analyzed according to the method of Litchfield and Wilcoxon (1949) to determine LC50's, variations, slope functions, and 95-percent confidence intervals.

RESULTS

Effects of QdSO₄ on test solutions

Stock solutions of QdSO₄ are acidic and influence the pH of bioassay water, especially softer waters. Stock solutions containing 75 grams of QdSO₄ in a liter of deionized water have a pH of 1.5. Since different amounts of QdSO₄ are added to bioassays to obtain data on the survival and mortality of fish, the chemical properties of the test solutions must be considered. Concentrations ranging from 0 to 80 mg/l of QdSO₄ were prepared in waters of various hardnesses, and the pH's were recorded (table 3). The pH in very soft water drops from 6.55 to 3.86 with the addition of 80 mg/l of QdSO₄. In harder waters which have a higher buffer capacity, the pH is more stable and decreases only one pH unit. These factors must be considered when assessing the toxicity of QdSO₄ to fish.

TABLE 3.--The influence of quinaldine sulfate on the pH of hard and soft water test solutions

Quinaldine sulfate (mg/l)	pH of different test solutions			
	Very soft	Soft	Hard	Very hard
0	6.55	7.10	7.78	8.00
5	6.29	6.92	7.57	7.82
20	5.65	6.61	7.30	7.55
40	5.40	6.22	7.00	7.39
60	4.02	6.04	6.87	7.19
80	3.86	5.67	6.61	7.01

Species and sizes of fish

Among the coldwater species, lake trout are the most sensitive to QdSO₄ at all exposure periods (LC50 = 26.3 mg/l at 3 hours and 15.5 mg/l at 96 hours) (table 4). Brook trout are the most resistant in 3-hour exposures (LC50 = 83.0 mg/l), while coho salmon are the most resistant at 96 hours (LC50 = 32.8 and 42.0 mg/l).

Among the warmwater species, largemouth bass are the most sensitive to QdSO₄ (96-hour LC50 = 6.80 mg/l). The most highly resistant species, carp and black bullhead, have 96-hour LC50's of 72.5 and 72.0 mg/l, respectively (table 4). In 96-hour exposures, largemouth bass and walleye are more sensitive than lake trout, and green sunfish are more sensitive than rainbow trout or brown trout.

Effects of temperature and water hardness

Rainbow trout are more susceptible to the toxic properties of QdSO₄ in cold water than in warm water in exposures of 1 to 6 hours (table 5). This trend reverses in 96-hour exposures, however, and the anesthetic is more toxic at 17° C. than at 7° or 12° C. At 24 hours the LC50 is 23.8 mg/l for 7° and 17° C., but at 12° C. the trout are more resistant (LC50 = 37.0 mg/l). Warmer temperatures cause greater variations in toxicity. At 17° C. the LC50 decreases from 47.0 at 1 hour to 12.8 mg/l at 96 hours, while at 7° C. the LC50 decreases from 28.9 at 1 hour to 23.8 mg/l at 96 hours. The most significant

TABLE 4.--Toxicity of quinaldine sulfate to fish in standard reconstituted water at 12° C.

Species	Average length (cm)	Average weight (grams)	LC50 and 95-percent confidence interval (mg/l) at			
			3 hours	6 hours	24 hours	96 hours
Coho salmon (<i>Oncorhynchus kisutch</i>)	4.1	0.7	80.0 55.1-116	80.0 55.1-116	47.5 41.9-53.9	32.8 30.0-35.9
"	11.4	17.0	---	70.0 52.1-94.0	45.0 42.1-48.1	42.0 37.6-46.9
Rainbow trout (<i>Salmo gairdneri</i>)	4.1	0.6	41.1 38.0-44.5	40.0 36.1-44.3	37.0 32.5-42.2	31.8 28.7-35.2
Brown trout (<i>Salmo trutta</i>)	3.8	0.6	55.0 47.0-64.4	---	32.7 28.5-37.5	28.3 25.7-31.2
Brook trout (<i>Salvelinus fontinalis</i>)	4.8	1.2	83.0 55.5-124	59.0 46.6-74.7	27.2 23.7-31.3	22.2 19.5-25.2
"	13.7	31.0	39.0 36.2-42.1	36.0 30.8-42.1	20.0 16.3-24.5	20.0 16.3-24.5
Lake trout (<i>Salvelinus namaycush</i>)	4.1	0.5	26.3 23.1-30.0	23.4 20.8-26.3	16.3 14.3-18.6	15.5 13.7-17.5
Carp (<i>Cyprinus carpio</i>)	4.6	1.4	85.4 73.2-99.7	80.0 64.9-98.6	74.6 59.3-93.8	72.5 59.7-88.1
Black bullhead (<i>Ictalurus melas</i>)	4.6	1.2	---	---	102 66.7-156	72.0 57.6-90.0
Channel catfish (<i>Ictalurus punctatus</i>)	5.6	1.5	60.0 53.7-67.1	49.4 43.3-56.4	39.1 34.0-45.0	32.9 27.4-39.5
Green sunfish (<i>Lepomis cyanellus</i>)	4.3	1.5	---	---	23.5 21.8-25.4	23.5 21.8-25.4
Bluegill (<i>Lepomis macrochirus</i>)	4.3	1.5	44.1 39.4-49.4	42.3 37.4-47.2	36.8 32.5-44.7	32.0 27.3-37.5
Largemouth bass (<i>Micropterus salmoides</i>)	4.6	1.4	20.8 18.3-23.5	19.4 17.1-22.0	16.0 9.64-26.7	6.80 3.71-13.6
Walleye (<i>Stizostedion vitreum</i>)	5.3	1.3	17.9 15.9-20.1	17.9 15.9-20.1	17.2 15.4-19.2	15.0 12.1-18.5

increase in toxicity is at 17° C. in 24 and 96 hours.

Rainbow trout are least susceptible to the toxic effects of QdSO₄ in very soft water (12 mg/l of total hardness) (table 5). Extremely high concentrations of the anesthetic are necessary in very soft water to kill the trout in 1- to 6-hour exposures. None of the trout died when exposed for 1 hour to 140 mg/l of the drug. The decreased activity of QdSO₄ in very soft water can be attributed to a decrease in pH as indicated in table 3. At 140 mg/l of QdSO₄, the pH of the test solution dropped to 3.35, which is below the pKa value of 5.42 (Knight et al., 1955; Sober, 1968). The equilibrium, therefore, is shifted in favor of the ionized form which is relatively unavailable to the fish (Sills and Allen, 1971). The extreme pH condition perhaps contributed to the mortality by stressing the fish.

In soft water (44 mg/l of total hardness), QdSO₄ is considerably more toxic than in very soft water, and LC50's range from 46.1 mg/l at 1 hour to 31.8 mg/l at 96 hours (table 5). In hard water (170 mg/l of total hardness) and in very hard water (300 mg/l of total hardness), the toxicity of QdSO₄ to rainbow trout is insignificantly different at each exposure (table 5). Also, the LC50's change very little in 1- to 96-hour exposures at each water hardness, but the values clearly show that QdSO₄ is consistently more toxic to trout in harder than in softer water at 12° C.

Effects of buffered and aged solutions of QdSO₄

Selected pH's ranging from 6 to 10 were produced in soft water (44 mg/l of total hardness) with the buffering agents listed in table 2. These

TABLE 5.--Toxicity of quinaldine sulfate to rainbow trout at different temperatures and water hardness

Water hardness	Temp. (°C.)	LC50 and 95-percent confidence interval (in mg/l) at				
		1 hour	3 hours	6 hours	24 hours	96 hours
soft ¹	7	28.9 24.3-34.4	28.5 24.7-32.1	25.8 22.6-29.4	23.8 20.3-27.9	23.8 20.3-27.9
soft ¹	12	46.1 41.4-51.4	41.1 38.0-44.5	40.0 36.1-44.3	37.0 32.5-42.2	31.8 28.7-35.2
soft ¹	17	47.0 39.5-55.9	46.0 40.6-52.1	42.5 35.0-51.7	23.8 19.2-29.5	12.8 11.1-14.9
very soft	12	---	133 116-153	90.5 83.2-98.4	65.5 62.2-69.0	50.5 46.0-55.5
hard	12	30.0 27.3-32.9	29.6 28.3-31.0	29.6 28.3-31.0	23.5 21.3-25.9	22.9 21.9-24.9
very hard	12	28.9 26.0-32.1	28.2 24.8-32.0	28.2 24.8-32.0	25.0 21.9-28.5	23.0 20.1-26.3

¹ Standard reconstituted water used in routine bioassays.

data were analyzed at 24 hours because QdSO₄ does not kill the trout in shorter exposures in the acid waters tested, and buffer chemicals complicate the toxicity of chemicals to rainbow trout at very high and very low pH levels in longer exposures (Marking, 1969). Despite the buffering capacity of these solutions, the pH decreased proportionately with concentrations, and the changes were greatest at pH 6. For instance, 20.0 mg/l of QdSO₄ in water buffered to pH 6 decreased the

pH to 5.85, whereas 400 mg/l of the anesthetic in that water decreased the pH to 3.30.

The 400-mg/l concentration did not kill any rainbow trout at 6 hours, but they all died within 24 hours. Again, we suspect that the fish were stressed by the low pH. The QdSO₄ is more toxic in near neutral or basic water than in acid water (table 6), and LC50's range from 20.7 to 62.0 mg/l at pH 10 and 6, respectively. The most

TABLE 6.--Toxicity of quinaldine sulfate to rainbow trout in pH buffered water, in pH readjusted buffered water, and in aged (1 week) pH readjusted buffered water at 12° C.

pH (original)	24-hour LC50 and 95-percent confidence interval in mg/l in		
	pH buffered tests (fresh)	pH readjusted tests ¹ (fresh)	pH readjusted tests ¹ (aged)
6.0	62.0 54.1-71.1	47.1 40.7-54.5	40.0 33.0-48.6
² 7.4	37.0 32.5-42.2	26.3 23.5-29.4	28.2 25.6-31.1
8.0	23.2 20.0-27.0	24.4 21.0-28.3	23.8 20.6-27.6
9.0	25.0 22.7-27.5	25.7 22.4-29.4	23.8 21.6-26.2
10.0	20.7 18.5-23.2	21.1 18.0-24.8	15.9 13.5-18.8

¹ Readjusted to desired pH at all concentrations of quinaldine sulfate.

² Standard reconstituted water.

significant decrease in activity is from pH 7.4 to pH 6.

In tests in alkaline waters of pH 8, 9, and 10, where the pH was readjusted to original buffered pH's at all concentrations of QdSO₄, the toxicity was insignificantly different than unadjusted tests (table 6). The toxicity of QdSO₄ increased in near neutral and acid waters when the pH was readjusted to 7.4 and 6.0, and LC50's are 26.3 and 47.1 mg/l, respectively. Again, QdSO₄ is more toxic in high pH waters.

Concurrent with the fresh, pH readjusted tests, similar concentrations of QdSO₄ were pH readjusted and aged for 1 week prior to introducing rainbow trout into the bioassays. The aged, pH readjusted solutions of QdSO₄ remain as active as fresh, pH readjusted solutions at the pH's tested (table 6). In fact, the aged, pH readjusted solutions are significantly more toxic at pH 10.

DISCUSSION

Quinaldine sulfate is consistently less toxic to fish than quinaldine (Marking, 1969). The 96-hour LC50's for QdSO₄ when tested against brook trout and channel catfish were 20.0 and 32.9 mg/l, respectively. The 96-hour LC50's for quinaldine were 12.0 mg/l for brook trout and 19.9 mg/l for channel catfish. However, when the concentrations are computed on the basis of active ingredients, the formulations are approximately equal in activity.

The activity of QdSO₄ is similar at various temperatures to that of quinaldine (Marking, 1969). Both compounds are more toxic at colder temperatures in 1- to 6-hour exposures, but the trend begins to change at 24 hours and reverses at 96 hours for the extreme temperatures. These data suggest that exposures at warmer temperatures are perhaps safer (less toxic), providing the exposures are short. In contrast, MS-222 (a fish anesthetic) is more toxic to fish at warmer temperatures (Marking, 1967).

Tests in very soft and acid water indicate that the activity of QdSO₄ is decreased considerably in these waters. In agreement, Schoettger and Julin (1969) and Sills and Allen (1971) report that quinaldine loses its effectiveness in solutions

having pH values less than 6. Since rainbow trout are generally intolerant to high or low pH's, we suspect that the low pH is more responsible for mortality than the QdSO₄ in this water.

CONCLUSIONS

1. QdSO₄ is toxic to fish, and the 96-hour LC50's for 12 species in standard reconstituted water range from 6.80 to 72.5 mg/l. Carp are most resistant, while lake trout are most sensitive.
2. QdSO₄ is more toxic to rainbow trout in cold water than in warm water in 1- to 6-hour exposures, but the trend reverses at 96 hours.
3. The anesthetic is more toxic in hard than in soft water, but the increased pH in hard water perhaps contributes to the greater toxicity.
4. Higher concentrations of QdSO₄ decrease the pH of buffered and nonbuffered test solutions significantly, thereby decreasing the concentration of the un-ionized quinaldine. We suspect that the low pH stressed the fish and contributed to the mortality. The anesthetic is consistently toxic in buffered solutions of pH 8, 9, and 10, but the drug is considerably less toxic at pH 6.
5. The anesthetic in reconstituted water remains as toxic in solutions aged for 1 week as in fresh solutions.

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49. The Efficacy of Quinaldine Sulfate as an Anesthetic for Freshwater Fish

By Philip A. Gilderhus, Bernard L. Berger,
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THE EFFICACY OF QUINALDINE SULFATE AS AN ANESTHETIC FOR FRESHWATER FISH

By Philip A. Gilderhus, Bernard L. Berger, Joe B. Sills,
and Paul D. Harman

ABSTRACT.--Quinaldine sulfate (QdSO_4), an improved formulation of the fish anesthetic quinaldine, was tested for its efficacy on 15 species of freshwater fish. The new crystalline formulation is water soluble and thus easier to use than is practical grade quinaldine. QdSO_4 anesthetizes most salmonids at 25 mg/l in less than 4 minutes and the fish recover in 1 to 13 minutes in fresh water. The effective concentrations for several warm-water species varied from 15 mg/l for bluegills to 60 mg/l for channel catfish. Fish were held for up to 60 minutes in effective concentrations without suffering mortalities. The efficacy of the anesthetic was little affected by water temperature, but the compound lowers the pH of some soft waters to below 6, the point at which it becomes ineffective as an anesthetic. All fish retained some reflex action and thus, some large fish were difficult to handle.

INTRODUCTION

Quinaldine (2-methylquinoline) has been used to anesthetize fish for spawning, marking, measuring, and long-distance transport of fish. Schoettger and Julin (1969) demonstrated that practical grade quinaldine anesthetizes several species of coldwater and warmwater fish to a handleable condition at 15 to 70 mg/l in 2 minutes or less. Fish were held for up to 6 hours in effective concentrations and recovered in less than 1 hour after their return to fresh water.

Practical grade quinaldine (liquid, 95 percent), however, is insoluble in water and has a disagreeable odor. To overcome these disadvantages, Allen and Sills (1973) synthesized quinaldine sulfate (QdSO_4), a crystalline salt of quinaldine, which is water soluble and has little or no odor. Marking and Dawson (1973) have investigated the toxicity of quinaldine sulfate and found the material generally of comparable or lower toxicity than practical grade quinaldine (Marking, 1969) on an active

ingredient basis using fish of comparable size at the same water temperature.

The efficacy of QdSO_4 was tested on freshwater fish to provide guidelines for use by fishery workers and to provide the proof of efficacy required by the regulatory agencies for registration.

METHODS AND MATERIALS

The quinaldine sulfate (59.3 percent quinaldine) used in these tests was formulated at the Southeastern Fish Control Laboratory at Warm Springs, Georgia. This compound is an experimental drug and is not registered for use as an anesthetic for fish. Aliquots of anesthetic were weighed individually for each concentration or test vessel.

Depending on the availability of fish, the anesthetic was tested on some species at the Fish Control Laboratory, La Crosse, Wisconsin, on some at the Warm Springs laboratory, and on some at both laboratories. Field

tests were conducted at several fish hatcheries where fish were anesthetized during spawning operations.

Tests using fingerling-size fish were conducted in 15-liter glass jars. Larger fish were exposed to the anesthetic in the laboratory and field in 45- and 100-liter polyethylene tanks. Water temperatures were adjusted and maintained by placing the test vessel in a water bath of the appropriate temperature.

The species of fish exposed to the anesthetic were coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Salmo gairdneri*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), white amur (*Ctenopharyngodon idella*), white sucker (*Catostomus commersoni*), black bullhead (*Ictalurus melas*), channel catfish (*Ictalurus punctatus*), bluegill (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), and walleye (*Stizostedion vitreum*).

The fish for laboratory tests were obtained from Federal and State fish hatcheries or, in

the case of small coho salmon and rainbow trout, hatched and reared at the La Crosse laboratory. All fish tested in the laboratories were maintained as described by Hunn et al. (1968). The fish were acclimated to the test water and temperature for 16 to 24 hours before their exposure to the anesthetic.

The laboratory tests at La Crosse were conducted in well water and soft, reconstituted water. Those at Warm Springs were conducted in limed spring water (table 1). The field tests were conducted in the water supplies of the respective fish hatcheries (table 2).

TABLE 1.--Characteristics of waters used in laboratory tests of quinaldine sulfate as an anesthetic for fish

Source	Type	pH	Total hardness (mg/l)	Total alkalinity (mg/l)
Well--La Crosse	hard	7.6-7.9	238-258	218-236
Reconstituted--La Crosse	soft	7.2-7.6	40-48	30-35
Spring--Warm Springs	soft	6.8-7.0	20	---

TABLE 2.--Characteristics of hatchery water supplies used in tests of QdSO_4 to anesthetize adult fish

Location	Species tested	Water temp. ($^{\circ}\text{C}.$)	pH	Total alkalinity (mg/l)	Total hardness (mg/l)
Platte River SFH ¹ Michigan	coho salmon	6	7.8	150	168
Manchester NFH ² Iowa	rainbow trout	9	7.5	172	215
Manchester NFH Iowa	brown trout	8	---	---	---
Osceola SFH Wisconsin	brook trout	9	8.1	171	208
Crystal Springs SFH Minnesota	lake trout	8	7.5	257	280
Lansing SFH Iowa	northern pike	5	9.2	141	144
Lansing SFH Iowa	walleye	10	9.2	133	164

¹ State fish hatchery.

² National fish hatchery.

The temperatures of test solutions were 7°_b, 12°_b, 17°_b, 22°_b, and 27°_b C. at La Crosse and 19°_b C. at Warm Springs. Field tests were conducted at the existing temperatures of the hatchery water supplies.

Schoettger and Julin (1967) defined loss of equilibrium, Stage 2, as the stage of anesthesia at which locomotion ceases, opercular rate slows, but reflex response to pressure on the caudal fin or peduncle is retained. In their tests with quinaldine, Schoettger and Julin (1969) found that the depth of anesthesia in fish rarely progresses beyond loss of equilibrium, Stage 2. Therefore, the majority of our tests were designed to define the most economical concentrations which would anesthetize 100 percent of the individual fish exposed, to loss of equilibrium, Stage 2, in 4 minutes or less. These criteria appear to satisfy the requirements for most of the field uses of fish anesthetics.

RESULTS

Reactions of fish

The reactions of fish to quinaldine sulfate were similar to their reactions to practical grade quinaldine as described by Schoettger

and Julin (1969). Initially the fish show a short period of increased activity, but the anesthetization progresses rapidly to loss of equilibrium, Stage 2, and rarely progresses beyond that stage.

The fish usually retained reflex action which could be triggered by tactile stimuli. The reflex action in large adult fish sometimes made them difficult to handle without injury to the fish. Northern pike were most likely to display this action and salmonids the least.

Efficacy of the anesthetic

Twenty to 30 mg/l of QdSO₄ anesthetized the five species of salmonids to loss of equilibrium, Stage 2, in approximately 4 minutes or less (table 3). Twenty-five mg/l of QdSO₄ (15 mg/l of quinaldine) were effective on most species and sizes of salmonids, comparing favorably with the 15 to 16 mg/l of quinaldine found to be effective by Schoettger and Julin (1969). All of the salmonids in our tests recovered in fresh water within 1 to 13 minutes compared to 3 to 21 minutes for salmonids in similar exposures to quinaldine as stated by Schoettger and Julin (1969). Fish were retained up to 60 minutes in loss of equilibrium, Stage 2, without any apparent harm.

TABLE 3.--Efficacy of quinaldine sulfate as an anesthetic for salmonids in laboratory tests

Species	Mean weight (grams)	Type of water	Temp. (°C.)	Concentration (mg/l)	Number of fish	Exposure time (min.)	Time in minutes to	
							Loss of equilibrium Stage 2	Recovery
Coho salmon	12	hard	12	20	15	15-60	0.9-1.3	1.0-2.8
Rainbow trout	450	hard	7	25	15	15	1.0-1.8	6.0-8.5
Do.	80	hard	12	25	15	15-60	1.0-2.0	2.5-5.7
Do.	25	hard	17	25	30	15-60	0.6-1.0	1.0-4.2
Brown trout	18	hard	7	25	15	15-60	0.7-1.0	5.0-10.0
Do.	16	hard	12	25	15	15-60	0.9-1.2	3.5-8.5
Do.	16	hard	17	25	15	15-60	0.5-0.9	3.4-6.0
Brook trout	27	hard	7	25	15	15-60	1.0-1.6	3.0-8.0
Do.	27	hard	12	25	15	15-60	2.0-3.0	2.3-6.0
Do.	1.1	soft	12	25	5	15	1.1-1.2	2.2-3.0
Lake trout	25	hard	7	25	10	15-30	0.9-1.2	8.0-13.0
Do.	30	hard	12	25	15	15-60	0.8-1.5	3.5-8.0
Do.	25	hard	17	25	10	15-30	0.7-1.2	2.2-5.1

The same concentrations of QdSO₄ which were effective in the laboratory (20 to 25 mg/l) were effective on larger trout and salmon in field tests (table 4). The large fish exhibited stronger reflex actions when touched or gently squeezed.

The warmwater species generally required higher concentrations of the anesthetic than did the salmonids. Northern pike, black bullheads, and channel catfish were the most resistant fishes requiring up to 65 mg/l of QdSO₄ for rapid anesthetization (table 5). Bluegills were the most susceptible, being anesthetized by 15 to 25 mg/l. On an active ingredient basis our 9 to 15 mg/l for bluegills and 15 mg/l for largemouth bass compare very favorably with the 15 mg/l found to be effective on both species by Schoettger and Julin (1969). The warmwater fish recovered after 1 to 22 minutes in fresh water.

The speed at which a given concentration of QdSO₄ induced anesthesia was somewhat related to the size of fish, with larger fish requiring slightly longer times to reach the same stage (table 6). The differences were minimal and would not usually justify the use of higher concentrations on larger fish. When the size of fish was constant, higher concentrations sometimes induced anesthesia more rapidly. However, reducing the time needed for anesthetization by 1 to 2 minutes does not generally warrant the use of greatly increased concentrations.

The water temperature, in most cases, did not appreciably affect the efficacy of the anesthetic. Among salmonids, a single concentration was usually effective on any particular species at all temperatures. Northern pike and black bullheads were anesthetized by somewhat lower concentrations at temperatures of 22° to 27° C. than at 12° C.

TABLE 4.--Efficacy of quinaldine sulfate as an anesthetic for adult fish at field installations

Species and location	No. of fish	Average weight of fish (kg)	Effective concentration (mg/l)	Time in minutes to	
				Loss of equilibrium Stage 2	Recovery in fresh water
Coho salmon Platte River SFH ¹ , Michigan	3	3.5	25	3.3	3.5
Rainbow trout Manchester NFH ² , Iowa	12	0.3	25	0.9-1.5	3.0-4.0
Brown trout Manchester NFH, Iowa	21	1.1	25	0.8-1.2	5.3-8.3
Brook trout Osceola SFH, Wisconsin	31	0.7	25	0.9-2.3	2.0-4.5
Lake trout Crystal Springs SFH, Minnesota	6	1.5	25	2.5-3.0	---
Northern pike Lansing SFH, Iowa	9	1.6	30	6.0-11.5	9.8-22.0
Walleye Lansing SFH, Iowa	3	0.8	20	2.1-3.2	3.0-18.8

¹ State fish hatchery.

² National fish hatchery.

TABLE 5.--Efficacy of quinaldine sulfate as an anesthetic for warmwater fishes in laboratory tests

Species	Mean weight (grams)	Type of water	Temp. (°C.)	Concentration (mg/l)	Number of fish	Exposure time (min.)	Time in minutes to	
							Loss of equilibrium Stage 2	Recovery
Northern pike	115	soft	7	40	5	15	3.3-4.3	10.0-11.7
Do.	115	hard	12	40	5	15	2.5-4.2	4.0-6.1
Do.	115	soft	17	40	5	15	1.3-2.6	2.0-3.0
Do.	115	soft	22	25	5	15	2.5-2.9	0.9-1.1
Carp	60	hard	12	35	15	15-60	2.3-2.9	6.3-15.9
Do.	387	hard	22	25	10	5.5-15	1.7-4.5	2.5-5.0
Do.	387	hard	27	25	5	15	3.6-3.8	1.5-6.2
White amur	227	soft ¹	19	30	3	30	1.1-1.6	4.0-6.0
Black bullhead	208	hard	12	50	10	5.5-15	2.5-3.6	4.0-22.0
Do.	129	hard	22	25	10	5.5-15	2.8-3.7	1.7-4.3
Do.	129	hard	27	25	5	15	3.7	2.0-3.0
Channel catfish	1.8	hard	12	60	10	5.5-15	1.3-3.6	3.0-5.5
Do.	1.5	hard	17	60	5	15	0.9-1.1	1.8-6.2
Do.	1316	soft ¹	19	65	20	30	2.0-3.0	3.0-8.0
Bluegill	77	hard	17	15	5	15	1.7-2.1	2.0-3.7
Do.	80	hard	27	25	10	5.5-15	0.7-1.2	2.0-2.5
Do.	142	soft ¹	19	20	43	30	2.0-3.0	1.0-3.0
Largemouth bass	12	hard	17	25	5	15	1.1-1.5	2.8-3.5
Do.	908	soft ¹	19	30	30	30	1.8-3.0	3.0-10.0

¹ Spring water - Warm Springs Laboratory.

TABLE 6.--Examples of differences in rate of anesthesia among different size fish exposed to quinaldine sulfate in well water

Species	Mean weight (grams)	Temp. (°C.)	Concentration (mg/l)	Time to loss of equilibrium, Stage 2 (min.)
Rainbow trout	450	12	25	0.8-1.5
Do.	1.5	12	25	0.5
Do.	450	17	25	0.8-1.3
Do.	25	17	25	0.6-0.9
Lake trout	25	17	25	0.7-1.2
Do.	1.5	17	25	0.5-0.6

The hardness and pH of the water have a pronounced effect on the efficacy of QdSO_4 . In soft, poorly-buffered water, the addition of 60 mg/l of QdSO_4 reduces the pH to 6.04 (Marking and Dawson, 1973). At that pH, a significant portion of the quinaldine ionizes and becomes unavailable to the fish (Schoettger and Julin, 1969; Sills and Allen, 1971). The relationship of hardness and pH to efficacy was demonstrated by comparing the efficacy on northern pike in two different waters at 12° C. Forty mg/l were effective in hard water and 60 mg/l were ineffective in soft water.

DISCUSSION

Quinaldine sulfate proved to be an effective anesthetic for all of the species of fish on which it was tested. The fish were anesthetized to loss of equilibrium, Stage 2, in about 4 minutes or less, and all species but channel catfish survived 60 minutes of exposure to effective concentrations. A rather narrow range of concentrations (20 to 35 mg/l) was effective on salmonids, cyprinids, and centrarchids. Higher concentrations (40 to 65 mg/l) were required for northern pike and the ictalurids. The anesthetic is effective over a wide range of temperatures (7° to 27° C.) and in soft and hard waters. The species of fish used in the tests of QdSO_4 represent a wide range of physiological characteristics. Thus, the concentrations of 20 to 65 mg/l and exposures of up to 1 hour should serve as guidelines for use on most species of fish.

The only limiting factor on the efficacy of QdSO_4 is a pH below 6, at which the compound ionizes and becomes ineffective. The anesthetic itself is acidic and concentrations of 20 mg/l in very soft water (total hardness of 10 to 13 mg/l) and 60 mg/l in soft water (total hardness of 40 to 48 mg/l) lower the pH to near or below that point (Marking and Dawson, 1973). If the pH of the anesthetic solution is excessively low, it should be buffered back to the range of 6.5 to 7.0 with NaHCO_3 .

Schoettger and Julin (1969) stated that 1 ml of quinaldine would anesthetize 94.5 kg of rainbow trout before the time to anesthetiza-

tion increased. The effective concentrations of QdSO_4 were comparable, on an active ingredient basis, to those of practical grade quinaldine given by Schoettger and Julin (1969). By inference then, the weight of fish which can be anesthetized per unit of active ingredient of quinaldine and QdSO_4 are likely to be similar.

The major assets of QdSO_4 , like those of quinaldine, are rapid anesthetization and a long safe-exposure time. In addition, QdSO_4 is more convenient to use than quinaldine because it does not have to be put into solution with an organic solvent. As with quinaldine, QdSO_4 does not completely inhibit reflex response. The reflex movements of large fish may be objectionable to some workers. A given degree of reflex response, however, may be a hindrance to one person and of no consequence to another.

CONCLUSIONS

1. Quinaldine sulfate is an effective anesthetic for most species of freshwater fish in concentrations of 20 to 65 mg/l.
2. The crystalline, water soluble material is convenient to handle and is as effective as practical grade quinaldine, on an active ingredient basis.
3. The efficacy of QdSO_4 is not greatly influenced by water temperature or size of fish.
4. In soft waters, high concentrations of QdSO_4 lower the pH of the solution, rendering the anesthetic ineffective.
5. The reflex action retained by fish under anesthesia may interfere with the handling of large fish.

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50. Residue of Quinaldine in Ten Species of Fish Following Anesthesia with Quinaldine Sulfate

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RESIDUE OF QUINALDINE IN TEN SPECIES OF FISH FOLLOWING ANESTHESIA WITH QUINALDINE SULFATE

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ABSTRACT.--The concentration and persistence of residues of the anesthetic quinaldine in five species of both coldwater and warmwater fishes were measured following treatment with the new formulation quinaldine sulfate. Quinaldine accumulated in relation to increasing temperature, treatment concentration, and length of exposure. Mean concentrations of quinaldine residues ranged from 1.18 to 8.31 $\mu\text{g/g}$ for the 0-hour withdrawal time. Initial residues were higher in warmwater species than in salmonids. Although a wide range of residue concentrations (0.80-9.34 $\mu\text{g/g}$) occurred immediately after exposure, these residues are dissipated rapidly when the fish are placed in fresh water. All fish tested after 24 hours of withdrawal in fresh water contained 0.01 $\mu\text{g/g}$ or less of quinaldine residue with the exception of the rainbow trout treated at 7° C.

INTRODUCTION

Quinaldine sulfate was prepared by Allen and Sills (1973) as an improved form of the fish anesthetic quinaldine. In order to register this formulation, the concentration and

persistence of residues in fish tissues must be known. Sills and Harman (1970) determined residue levels in striped bass (*Morone saxatilis*) following anesthesia with the quinaldine salt. In the present study, 10 species of coldwater and warmwater fishes (table 1) were anesthetized with quinaldine sulfate at various concentrations, temperatures, and times, and muscle tissue was analyzed for quinaldine residue.

TABLE 1.--Species of fish analyzed for quinaldine residues following anesthesia with quinaldine sulfate

Common Name	Scientific Name
Coho salmon	<u>Oncorhynchus kisutch</u>
Brown trout	<u>Salmo trutta</u>
Rainbow trout	<u>Salmo gairdneri</u>
Lake trout	<u>Salvelinus namaycush</u>
Brook trout	<u>Salvelinus fontinalis</u>
Northern pike	<u>Esox lucius</u>
Channel catfish	<u>Ictalurus punctatus</u>
Largemouth bass	<u>Micropterus salmoides</u>
Bluegill	<u>Lepomis macrochirus</u>
Walleye	<u>Stizostedion vitreum</u>

METHODS AND MATERIALS

Ten species of fish used in this study were treated with efficacious concentrations of quinaldine sulfate by Gilderhus et al. (1973). Temperatures of treatment ranged from 7° to 19° C., and exposure times ranged from 5.5 to 30 minutes. A wide range of concentrations (20 to 65 mg/l) was necessary because of the variety of species and temperatures involved.

Withdrawal times began when exposed fish were placed in fresh, flowing water for recovery. Three fish were used for residue analysis at 0, 1, 2, 4, either 6 or 8, and 24 hours. Samples of muscle tissue were obtained from homogenized fillets (Luhning and Harman, 1971) and were analyzed for quinaldine by gas chromatography using the methods of Allen and Sills (1970a and 1970b). Three samples were analyzed at each withdrawal. Quinaldine residues were measured to 0.01 $\mu\text{g/g}$, and any value less than this was considered zero.

RESULTS

Coho salmon

Coho salmon were the largest fish tested (table 2). They were treated with 25 mg/l of quinaldine sulfate at 4° C. Mean quinaldine residues for the 0-hour withdrawal interval were 1.18 and 1.78 $\mu\text{g/g}$ for the 5.5- and 15-minute exposures, respectively. Quinaldine residue was less than 0.01 $\mu\text{g/g}$ for both ex-

TABLE 2.--Residues of quinaldine in coho salmon muscle following anesthesia with 25 mg/l of quinaldine sulfate at 4° C.

Withdrawal interval	Exposure time (minutes)	Mean weight (kg)	Residues ($\mu\text{g/g}$) ¹ Quinaldine	
			Mean	Range
control	0	2.03	0.00 ²	0.00
0-hour	5.5	4.07	1.18	1.03-1.26
1-hour	5.5	2.79	0.09	0.07-0.14
2-hour	5.5	3.83	0.03	0.01-0.06
4-hour	5.5	3.86	0.01	0.00-0.01
8-hour	5.5	3.25	0.00	0.00
24-hour	5.5	3.77	0.00	0.00
0-hour	15	3.96	1.78	1.03-2.46
8-hour	15	3.63	0.00	0.00
24-hour	15	2.69	0.00	0.00

¹ Each mean value represents the average of three analyses.

² Values less than 0.01 $\mu\text{g/g}$ are reported as 0.00.

posures after 24 hours of withdrawal from the anesthetic.

Brown trout

Brown trout were exposed to 25 mg/l of quinaldine sulfate at 12° C. for both 5.5 and 15 minutes. Quinaldine residues ranged from a mean of 1.44 $\mu\text{g/g}$ after 5.5 minutes of exposure to 1.67 $\mu\text{g/g}$ after 15 minutes of exposure. No detectable residue (less than 0.01 $\mu\text{g/g}$) remained after 8 hours of withdrawal from the anesthetic (table 3).

TABLE 3.--Residues of quinaldine in muscle of brown trout after anesthesia with 25 mg/l of quinaldine sulfate at 12° C.

Withdrawal interval	Exposure time (minutes)	Mean weight (grams)	Residues ($\mu\text{g/g}$) ¹ Quinaldine	
			Mean	Range
control	0	582	0.00 ²	0.00
0-hour	15	646	1.67	1.32-2.14
1-hour	15	525	0.65	0.48-0.86
2-hour	15	521	0.09	0.05-0.14
4-hour	15	584	0.02	0.02-0.02
8-hour	15	592	0.00	0.00
24-hour	15	515	0.00	0.00
0-hour	5.5	542	1.44	1.21-1.81
2-hour	5.5	555	0.03	0.02-0.04
8-hour	5.5	492	0.00	0.00

¹ Each mean value represents the average of three analyses.

² Values less than 0.01 $\mu\text{g/g}$ are reported as 0.00.

Rainbow trout

Rainbow trout were tested most extensively. Fish weighing from approximately 100 to 550 grams which had been exposed to 25 mg/l of quinaldine sulfate were analyzed for quinaldine residue. At 12° C., fish exposures to the anesthetic were 5.5 and 15 minutes. At 7° and 17° C. the fish were exposed to the anesthetic for 15 minutes (table 4). A quinaldine residue was detectable immediately following exposure to quinaldine sulfate and the initial residue was higher as the temperature and exposure time increased.

TABLE 4.--Residues of quinaldine in muscle of rainbow trout anesthetized with 25 mg/l of quinaldine sulfate at three temperatures

Withdrawal interval	Treatment		Mean Weight (grams)	Residues ($\mu\text{g/g}$) ¹	
	Exposures (minutes)	Temp. ($^{\circ}\text{C.}$)		Mean	Range
control	0	7	384	² 0.00	0.00
0-hour	15	7	419	3.53	2.40-4.80
1-hour	15	7	335	1.99	1.20-3.22
2-hour	15	7	480	0.67	0.64-0.72
4-hour	15	7	452	0.64	0.46-0.75
24-hour	15	7	547	0.17	0.15-0.18
control	0	12	135	0.00	0.00
0-hour	15	12	452	2.62	0.80-5.83
1-hour	15	12	460	0.73	0.46-1.28
2-hour	15	12	357	0.22	0.16-0.26
4-hour	15	12	327	0.04	0.01-0.06
8-hour	15	12	403	0.00	0.00
24-hour	15	12	455	0.00	0.00
0-hour	5.5	12	142	1.80	1.10-2.40
1-hour	5.5	12	131	0.32	0.30-0.33
2-hour	5.5	12	145	0.10	0.08-0.11
4-hour	5.5	12	118	0.03	0.03-0.03
8-hour	5.5	12	93	0.01	0.01-0.01
24-hour	5.5	12	111	0.00	0.00
control	0	17	135	0.00	0.00
0-hour	15	17	392	4.51	2.08-6.25
1-hour	15	17	433	2.02	1.55-2.87
2-hour	15	17	440	1.44	0.77-2.17
4-hour	15	17	360	1.11	0.90-1.33
8-hour	15	17	466	0.44	0.38-0.54

¹ Each mean value represents the average of three analyses.

² Values less than 0.01 $\mu\text{g/g}$ are reported as 0.00.

The rainbow trout exposed to the anesthetic for 15 minutes at 7 $^{\circ}$ C. contained a mean quinaldine residue of 3.53 $\mu\text{g/g}$ at the 0-hour withdrawal and 0.17 $\mu\text{g/g}$ after 24 hours of withdrawal. Those treated at 12 $^{\circ}$ C. for 5.5

and 15 minutes contained a mean residue of quinaldine of 1.80 and 2.62 $\mu\text{g/g}$, respectively. After 24 hours of withdrawal at 12 $^{\circ}$ C., the quinaldine residue concentrations had fallen below 0.01 $\mu\text{g/g}$ in both the 5.5- and 15-minute exposures. The rainbow trout treated at 17 $^{\circ}$ C. contained a mean residue of 4.51 $\mu\text{g/g}$ of quinaldine at 0-hour withdrawal and 0.44 $\mu\text{g/g}$ quinaldine after 8 hours of withdrawal. A 24-hour withdrawal was not available for this set of samples.

Lake trout and brook trout

Lake trout exposed to the anesthetic at 12 $^{\circ}$ C. for 5.5 and 15 minutes had quinaldine residues ranging from 1.49 to 3.90 $\mu\text{g/g}$ at the 0-hour withdrawal, and were 0.01 $\mu\text{g/g}$ or less after 8 hours of withdrawal (table 5). Brook trout were treated for 5.5 minutes at 9 $^{\circ}$ C. and contained a mean quinaldine residue of 3.13 $\mu\text{g/g}$ at the 0-hour withdrawal and 0.01 $\mu\text{g/g}$ after 8 hours of withdrawal. No quinaldine residue was detected in any of these fish after 24 hours of withdrawal.

Northern pike

Northern pike treated with 30 mg/l of quinaldine sulfate for 30 minutes at 7 $^{\circ}$ and 12 $^{\circ}$ C. were analyzed for quinaldine residue (table 6). Those treated at 7 $^{\circ}$ C. contained a mean quinaldine residue of 4.80 $\mu\text{g/g}$ at the 0-hour withdrawal and less than 0.01 $\mu\text{g/g}$ of quinaldine residue after 24 hours of withdrawal. Those treated at 12 $^{\circ}$ C. contained a mean quinaldine residue of 4.40 $\mu\text{g/g}$ at the 0-hour withdrawal and 0.17 $\mu\text{g/g}$ after 4 hours of withdrawal. No samples were available for the 12 $^{\circ}$ C. treatment with longer than 4 hours of withdrawal.

Channel catfish, largemouth bass and bluegill

The warmwater species including channel catfish, largemouth bass, and bluegill were exposed to the highest concentrations of the anesthetic and at the highest temperature, 19 $^{\circ}$ C., for 30 minutes (table 7). This resulted in the highest quinaldine residues encountered

TABLE 5.--Residues of quinaldine in muscle of lake trout and brook trout after anesthesia with 25 mg/l of quinaldine sulfate

Withdrawal interval	Treatment		Mean weight (grams)	Residues ($\mu\text{g/g}$) ¹ Quinaldine	
	Exposure (minutes)	Temp. ($^{\circ}\text{C.}$)		Mean	Range
Lake trout					
control	0	12	1630	² 0.00	0.00
0-hour	5.5	12	1580	2.04	1.49-2.59
1-hour	5.5	12	1270	0.17	0.10-0.28
2-hour	5.5	12	1440	0.11	0.08-0.15
4-hour	5.5	12	1690	0.02	0.01-0.03
8-hour	5.5	12	1500	0.01	0.01-0.01
24-hour	5.5	12	1170	0.00	0.00
0-hour	15	12	1430	3.50	3.09-3.90
8-hour	15	12	1620	0.01	0.01-0.02
24-hour	15	12	1470	0.00	0.00
Brook trout					
control	0	9	300	0.00	0.00
0-hour	5.5	9	302	3.13	2.27-3.41
1-hour	5.5	9	344	0.27	0.22-0.30
2-hour	5.5	9	332	0.09	0.06-0.14
4-hour	5.5	9	297	0.03	0.02-0.04
8-hour	5.5	9	370	0.01	0.00-0.01
24-hour	5.5	9	307	0.00	0.00

¹ Each mean value represents the average of three analyses.² Values less than 0.01 $\mu\text{g/g}$ are reported as 0.00.

at the 0-hour withdrawal, but after 24 hours of withdrawal they were 0.01 $\mu\text{g/g}$ or less.

Walleye

Walleye treated with 20 mg/l of quinaldine sulfate for 30 minutes at 7° C. contained a mean quinaldine residue of 2.60 $\mu\text{g/g}$ at the 0-hour withdrawal and 0.04 $\mu\text{g/g}$ after 6 hours of withdrawal (table 8). No samples were available with longer than 6 hours of withdrawal.

TABLE 6.--Residues of quinaldine in muscle tissue of northern pike following anesthesia with 30 mg/l of quinaldine sulfate

Withdrawal interval	Treatment		Mean weight (kg)	Residues ($\mu\text{g/g}$) ¹	
	Exposure (minutes)	Temp. (°C.)		Mean	Range
control	0	7	1.43	² 0.00	0.00
0-hour	30	7	1.89	4.80	2.10-8.50
1-hour	30	7	1.18	1.10	1.00-1.20
2-hour	30	7	1.00	0.51	0.33-0.63
4-hour	30	7	1.06	0.06	0.05-0.08
24-hour	30	7	1.12	0.00	0.00
control	0	12	1.48	0.00	0.00
0-hour	30	12	1.91	4.40	2.80-5.40
2-hour	30	12	1.63	0.68	0.61-0.76
4-hour	30	12	1.43	0.17	0.05-0.42

¹ Each mean value represents the average of three analyses.² Values less than 0.01 $\mu\text{g/g}$ are reported as 0.00.

TABLE 7.--Residues of quinaldine in muscle of channel catfish, bluegill, and largemouth bass anesthetized with quinaldine sulfate for 30 minutes at 19° C.

Withdrawal interval	Treatment QdSO ₄ (mg/l)	Mean weight (grams)	Residues ($\mu\text{g/g}$) ¹	
			Mean	Range

Channel catfish

control	0	1,270	² 0.00	0.00
0-hour	65	1,320	8.31	7.79-9.34
1-hour	65	1,680	5.55	5.44-5.86
2-hour	65	680	1.68	1.60-1.76
4-hour	65	1,320	0.91	0.82-1.00
6-hour	65	1,090	0.26	0.15-0.40
24-hour	65	1,680	0.01	0.01-0.01

See footnotes at end of table.

TABLE 7.--Residues of quinaldine in muscle of channel catfish, bluegill, and largemouth bass anesthetized with quinaldine sulfate for 30 minutes at 19° C.--Continued.

Withdrawal interval	Treatment QdSO ₄ (mg/l)	Mean weight (grams)	Residues (μg/g) ¹ Quinaldine	
			Mean	Range
Bluegill				
control	0	115	0.00	0.00
0-hour	20	152	3.72	3.43-4.07
1-hour	20	180	0.52	0.46-0.63
2-hour	20	135	0.33	0.12-0.61
4-hour	20	165	0.14	0.03-0.22
6-hour	20	110	0.03	0.02-0.04
24-hour	20	112	0.00	0.00-0.01
Largemouth bass				
control	0	1,090	0.00	0.00
0-hour	30	1,000	6.07	5.04-7.20
1-hour	30	1,000	0.76	0.44-1.00
2-hour	30	1,040	0.20	0.17-0.21
4-hour	30	590	0.05	0.04-0.06
6-hour	30	730	0.05	0.03-0.06
24-hour	30	770	0.00	0.00

¹ Each mean value represents the average of three analyses.

² Values less than 0.01 μg/g are reported as 0.00.

TABLE 8.--Residues of quinaldine in muscle of walleye following anesthesia with 20 mg/l of quinaldine sulfate at 7° C.

Withdrawal interval	Treatment Exposure (minutes)	Mean weight (kg)	Residues (μg/g) ¹ Quinaldine	
			Mean	Range
Walleye				
control	0	0.81	0.00	0.00
0-hour	30	1.32	2.60	2.20-3.00
1-hour	30	1.92	1.30	1.10-1.60
2-hour	30	0.82	0.93	0.70-1.20
6-hour	30	0.82	0.04	0.02-0.07

¹ Each mean value represents the average of three analyses.

² Values less than 0.01 μg/g are reported as 0.00.

DISCUSSION

Residues of quinaldine dissipated rapidly from the muscle of all fish in this study. A representative curve for disappearance of quinaldine residue is shown in figure 1. The curves represent the averages of mean values obtained on coho salmon, rainbow trout, brown trout, brook trout, and lake trout exposed to 25 mg/l of quinaldine sulfate for 15 minutes at 12° C., and channel catfish, largemouth bass, and bluegill exposed to 65, 30, and 20 mg/l of quinaldine sulfate, respectively, for a period of 30 minutes at 19° C. The regression of the residues in both groups of fish is similar to that of MS-222 reported by Walker and Schoettger (1967).

The warmwater fish accumulated higher concentrations of residues than the salmonids. This appears to be directly related to higher concentrations of anesthetic, higher temperature, and longer exposure time. Though there was wide variation in residue concentrations at the 0-hour withdrawal time, residues in all fish, except rainbow trout treated at 7° C., were at undetectable levels within 24 hours. The rainbow trout treated at 7° C., the coldest temperature tested, contained a mean quinaldine residue of 3.53 μg/g at the 0-hour withdrawal and after 24 hours of withdrawal from the anesthetic had fallen to a mean of 0.17 μg/g.

CONCLUSIONS

1. The residues of quinaldine in all the species tested varied considerably at the 0-hour withdrawal depending on the concentration of anesthetic used, temperature, and exposure time. An increase in any of these parameters also increased residue concentration at the 0-hour withdrawal time.
2. The residue concentration fell below 0.01 μg/g after 24 hours of withdrawal in all cases, except the rainbow trout treated at 7° C.
3. The residues of quinaldine dissipated more slowly at the lower temperatures. After 24 hours of withdrawal at 7° C., the rainbow trout still contained an average of 0.17 μg/g of quinaldine residue.

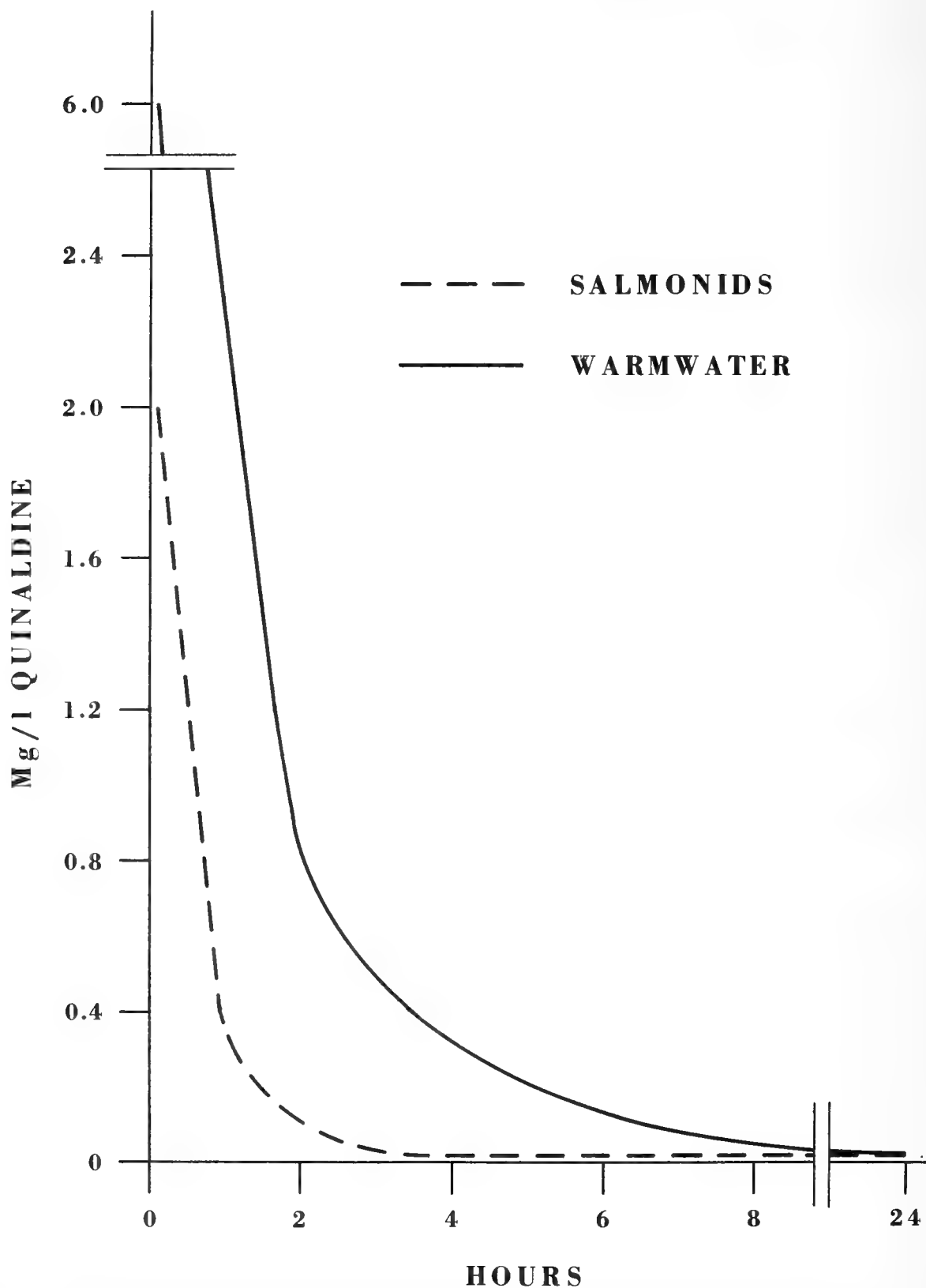


Figure 1.--Regression of quinaldine residues in muscle tissue of salmonids exposed to quinaldine sulfate at 12° C., and warmwater fish at 19° C., as a function of time.

4. Warmwater species had higher initial quinaldine residues than salmonids.

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- 51. Methods for Simultaneous Determination
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in Fish Tissues**
- 52. Residues of MS-222, Benzocaine, and Their Metabolites
in Striped Bass Following Anesthesia**



United States Department of the Interior
Fish and Wildlife Service
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By Charles W. Luhning

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METHODS FOR SIMULTANEOUS DETERMINATION AND IDENTIFICATION OF MS-222 AND METABOLITES IN FISH TISSUES

By Charles W. Luhning
Southeastern Fish Control Laboratory
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ABSTRACT.--MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is a primary aromatic amine commonly used to anesthetize fish. Like all primary aromatic amines, its diazonium salt reacts with N-1-naphthylethylenediamine dihydrochloride to form a wine-red azo dye with a maximum absorbance at 545 nm. Basic carbon tetrachloride extraction separates the azo dyes of MS-222 and its acid metabolite, m-aminobenzoic acid, and quantitative determination of each compound is made colorimetrically. By this method, recoveries of 82 to 110 percent for MS-222 and 84 to 117 percent for m-aminobenzoic acid were obtained from largemouth bass muscle and liver tissues spiked with 1 to 10 $\mu\text{g/g}$ of each compound. Confirmation of MS-222 and m-aminobenzoic acid residues in fish tissue was made by thin-layer chromatography. Quantitative estimation and identification of each compound were possible from samples spiked with as little as 1 $\mu\text{g/g}$ of MS-222 or m-aminobenzoic acid.

INTRODUCTION

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is a commonly used fish anesthetic. When any chemical or drug is used on a food commodity item, the U.S. Food and Drug Administration requires data on residual amounts of the drug and its metabolites left in the commodity after treatment. Previous methods of analysis for MS-222 residues in fish tissue did not identify m-aminobenzoic acid, a metabolite of MS-222. The method described by Walker and Schoettger (1967) included the residue of m-aminobenzoic acid, but the method did not separate it from MS-222 residues. The thin-layer chromatographic method of Allen, Luhning, and Harman (1970) only confirmed residues of MS-222 determined by the first method.

The analytical method described here will detect residues of MS-222 and its metabolites, namely, acetylated MS-222, m-aminobenzoic

acid and m-acetylaminobenzoic acid. The thin-layer chromatographic procedures are done simultaneously with the analytical procedures, which save time and materials.

METHODS AND MATERIALS

Reagents and apparatus

Reference to a company or product does not imply recommendation to the exclusion of others that may be suitable. Reagents and apparatus used were as follows:

1. Carbon tetrachloride, reagent grade.
2. Trichloroacetic acid: Dissolve 60 g crystalline TCA in distilled water. Transfer to a 100-ml volumetric flask and bring to volume with distilled water. Dilute 50 ml to 200 ml to obtain 15-percent TCA, and dilute 5 ml of the original stock to 100 ml to obtain 3-percent TCA.

3. 0.2-percent sodium nitrite: Transfer 0.2 g NaNO_2 into a 100-ml volumetric flask, dissolve, and bring to volume with distilled water. Make fresh daily.
4. 0.5-percent ammonium sulfamate: Transfer 0.5 g $\text{NH}_4\text{SO}_3\text{NH}_2$ into a 100-ml volumetric flask and bring to volume with distilled water.
5. 0.1-percent N-1-naphthylethylenediamine dihydrochloride: Transfer 0.1 g $\text{C}_{12}\text{H}_{14}\text{N}_2\cdot 2\text{HCl}$ into a 100-ml volumetric flask, dissolve, and bring to volume with distilled water. Refrigerate and make up fresh weekly.
6. 4N hydrochloric acid: Dilute 40 ml 10N HCl to 100 ml with distilled water.
7. 10-percent hydrochloric acid (V/V).
8. Concentrated ammonium hydroxide.
9. Standard solutions: Weigh 0.01 g of MS-222 and quantitatively transfer to a 100-ml volumetric flask, add 20 ml of 15-percent TCA and bring to volume with distilled water. One ml of this stock solution diluted to 100 ml with 3-percent TCA will make a 1 $\mu\text{g}/\text{ml}$ solution. Make fresh daily. Standards of m-aminobenzoic acid are made up weekly in like manner.
10. Ethyl acetate, reagent grade.
11. Iso-butanol, reagent grade.
12. Developing solutions: The first solution contains 180 ml ethyl ether, 10 ml acetone, and 10 ml concentrated glacial acetic acid. The second solution contains 120 ml chloroform, 38 ml ethyl alcohol, 38 ml methanol, and 4 ml concentrated ammonium hydroxide. All solvents are reagent grade or better.
13. Waring blender.
14. Tissue homogenizer, Virtis "45".
15. Orbital centrifuge.
16. Chromatography tank 10.2 by 20.3 by 22.9 cm, lined with absorbent paper.
17. Silica gel thin-layer plates, Eastman chromatogram sheets without fluorescent dye, 20 by 20 cm.
18. Chromatography spray bottle.
19. Micropipettes, 1, 5, 10, and 25 μl .
20. Centrifuge tubes, 15 and 40 ml.
21. Spectrophotometer cuvettes, round, 13 by 100 mm.
22. Spectrophotometer, Bausch and Lomb "Spectronic 20".
23. Whatman #40 filter paper, 9.0 cm diameter.
24. Funnels, 5 cm diameter.

Tissue collection and treatment

Blood samples are taken by caudal puncture (Steucke and Schoettger, 1967) with a heparinized syringe fitted with a 20-gauge needle. Place 1.0 ml of blood in a 40-ml graduated centrifuge tube, and add 15 ml of distilled water and 4 ml of 15-percent TCA. Mix thoroughly and centrifuge at 2,500 RPM for 30 minutes. Filter the supernatant through #40 Whatman filter paper, and the sample is ready for analysis. If a 1.0-ml sample of blood cannot be obtained, use the amount obtained and proportional amounts of water and TCA.

Fillet each fish after the other tissues have been removed, and homogenize the entire fillet in a Waring blender to obtain a homogeneous sample (Luhning and Harman, 1971). Weigh out 1.0 g of homogenized tissue in a tared homogenizing flask, add 10 ml of distilled water and homogenize for 1 minute. Quantitatively transfer to a 40-ml graduated centrifuge tube and bring to a volume of 16 ml with distilled water. Then add 4 ml of 15-percent TCA to coagulate the protein, mix thoroughly, and centrifuge at 2,500 RPM for 20 minutes. Filter the supernatant through #40 Whatman filter paper, and the sample is ready for analysis. Kidney, liver, and brain are dissected from the fish and prepared the same as muscle tissue, except that these samples are not homogenized before weighing out a 1.0-g sample.

Analytical procedures

The first seven steps are basically the same as those described by Walker and Shoettger (1967). The procedure is as follows:

1. Pipette 5 ml of 3-percent TCA into a clean 50-ml Erlenmeyer flask for a reagent blank.
2. Pipette 5 ml of 1- μ g/ml standard of MS-222 and m-aminobenzoic acid into Erlenmeyer flasks. Also pipette 5 ml of mixed MS-222 and acid standards, each at a 1- μ g/ml concentration, into an Erlenmeyer flask.
3. Pipette 5 ml of filtrate from each sample into separate 50-ml Erlenmeyer flasks.
4. Add 0.5 ml of 0.2-percent sodium nitrite to each flask, swirl, and let stand for 15 minutes. Keep samples at room temperature and out of direct sunlight.
5. Add 0.5 ml of 0.5-percent ammonium sulfamate to each flask, swirl, and let stand for 3 minutes.
6. Add 0.5 ml of 0.1-percent N-1-naphthylethylenediamine dihydrochloride to each flask, swirl vigorously, and let stand for 10 minutes.
7. Pour samples into a clean cuvette. Zero spectrophotometer at 100-percent transmittance using the reagent blank. Read percent transmittance of each sample in the spectrophotometer at 545 nm.
8. Transfer the azo dyestuff to a labeled 15-ml centrifuge tube containing 0.5 ml of 7.5 M ammonium hydroxide and 2 ml of carbon tetrachloride. Shake vigorously, and centrifuge at 1,500 RPM for 3 minutes.
9. Rinse each cuvette with distilled water and shake dry. Be sure to keep cuvettes in order. Add 1 ml of 60-percent TCA to each cuvette.
10. After centrifugation (step 8), transfer 5 ml of the aqueous layer back into the original cuvette and mix gently.

11. Zero the spectrophotometer at 100-percent transmittance using the extracted reagent blank, and again read percent transmittance of each sample with a spectrophotometer at 545 nm.

12. Convert all percent transmittance readings to absorbance, and calculate μ g/g residues as follows:

$$\mu\text{g/g free MS-222} = \frac{S_1 \times \frac{A_2}{A_1} - S_2}{M_1 \times \frac{A_2}{A_1} - M_2} \times 20$$

$$\mu\text{g/g free } \underline{m}\text{-aminobenzoic acid} = \frac{S_2}{A_2} \times 20$$

S_1 = absorbance of sample before extraction.

S_2 = absorbance of sample after extraction.

M_1 = absorbance of mixed standard (1 μ g/ml MS-222 and 1 μ g/ml m-aminobenzoic acid) before extraction.

M_2 = absorbance of mixed standard after extraction.

A_1 = absorbance of m-aminobenzoic acid standard (1- μ g/ml) before extraction.

A_2 = absorbance of m-aminobenzoic acid after extraction.

13. Possible acetylated derivatives can be analyzed as follows:

- A. Place 5 ml of filtrate from each sample into separate 15-ml graduated centrifuge tubes and add 0.5 ml of 4N HCl.
- B. Place tubes in a boiling water bath for 1 hour, cool and adjust volume to 5 ml with distilled water.

- C. Transfer to a 50-ml Erlenmeyer flask and analyze as before, starting with step 4.
 - D. The amount of acetylated residues is obtained by subtracting the concentration of the first analysis (free residues) from the concentration of the second analysis.
14. For the identification and confirmation of MS-222 and *m*-aminobenzoic acid residues by thin-layer chromatography, proceed as follows:
- A. React another 5 ml of filtrate from each sample and standards with the Bratton-Marshall reagents (steps 1 through 6).
 - B. Transfer the azo dyestuff to a stoppered test tube containing 1 ml of iso-butanol-ethyl acetate (50/50, V/V). Shake vigorously, and let stand for 30 minutes in a dark place.
 - C. Mark a spotting line 2.5 cm and a solvent-front line 12.5 cm from the bottom of a 20 by 20 cm silica gel thin-layer plate.
 - D. With a calibrated micropipette, spot enough iso-butanol-ethyl acetate on spotting line to give a visible, colored spot for each sample. On the same plate, spot 1, 2, and 5 μ l of the mixed standard (1- μ g/ml concentration of each).
 - E. Thirty minutes before developing the plate, mix 180 ml of ethyl ether, 10 ml of acetone, and 10 ml of concentrated acetic acid (90:5:5) and pour into a chromatography tank lined with absorbent paper. Place the plate in the tank and allow the solvent to rise to the top of the plate, then remove and dry under an electric hair dryer in a fume hood.
 - F. Develop the plate again in 120 ml of chloroform, 38 ml of ethanol, 38 ml

of methanol, and 4 ml of concentrated ammonium hydroxide (60:19:19:2). Allow the solvent front to migrate up to the previously marked line (12.5 cm from the bottom), then remove the plate, dry, and spray with 10-percent HCl under a fume hood.

- G. Compare the R_f values of the standards to those of the samples. In general, the R_f values will be 0.83 for MS-222 and 0.18 for *m*-aminobenzoic acid. An estimate of the concentration present in the samples can be made by comparing the color intensity and size of the sample spot to that of a spot obtained from a known amount of standard.

A flow chart has been prepared to diagram the sequence of the above procedures (fig. 1). The chemical structures of MS-222 and its metabolites are illustrated in figure 2.

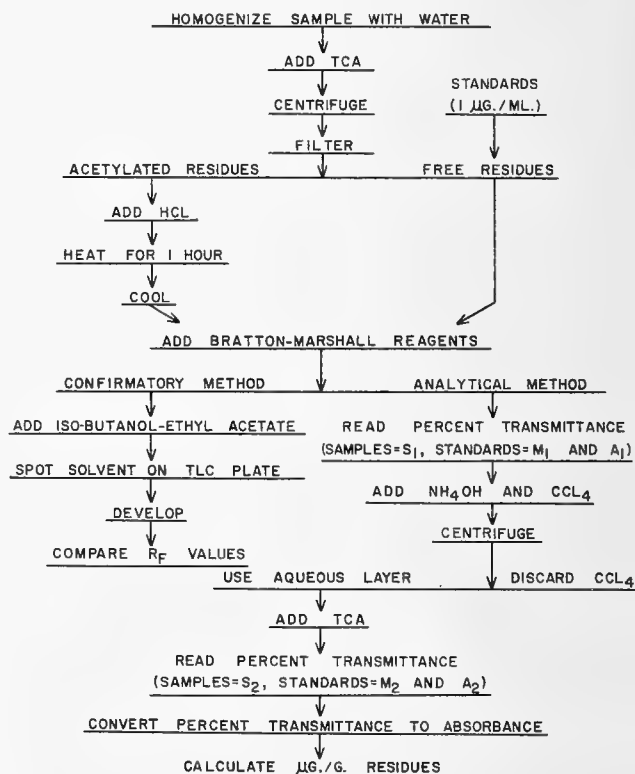


Figure 1.--Flow chart of procedures for the determination and identification of MS-222 and metabolites.

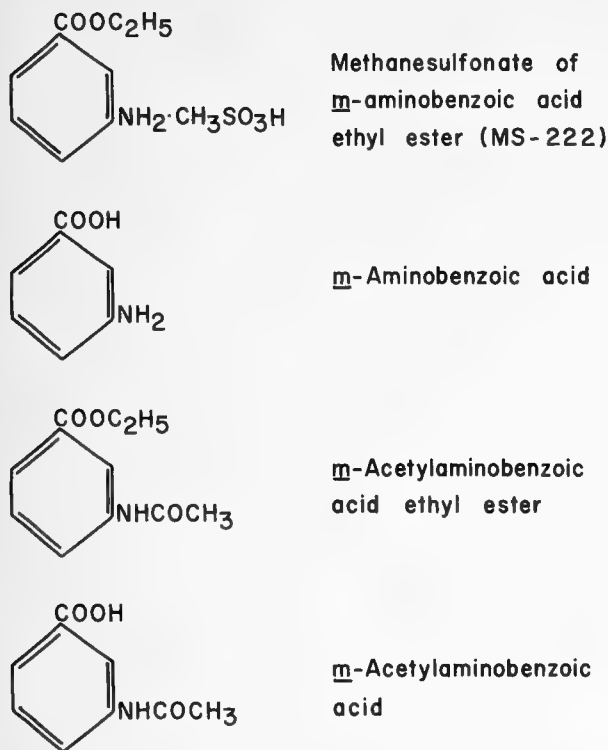


Figure 2.--Chemical structures of MS-222 and metabolites.

RESULTS

The efficiency of the extraction procedure was evaluated by analyzing a series of pure and mixed standards of MS-222 and m-aminobenzoic acid before and after extraction with basic carbon tetrachloride (fig. 3). The concentration of the standard did not influence the amount extracted, for the regression of absorbance values was relatively linear with increasing concentration of the standards. The absorbance value for the pure m-aminobenzoic acid standard before extraction, divided by the absorbance value after extraction (A_2/A_1) does not equal the known dilution factor of the procedure (D). The difference is attributed to a small loss of material left in the cuvette after transferring the material to a centrifuge tube. Thus, in figure 3, the slope of the absorbance curve for MS-222 obtained from the mixed standards ($M_1 \times A_2/A_1 - M_2$) is slightly different from one calculated by the known dilution factor (MS X D).

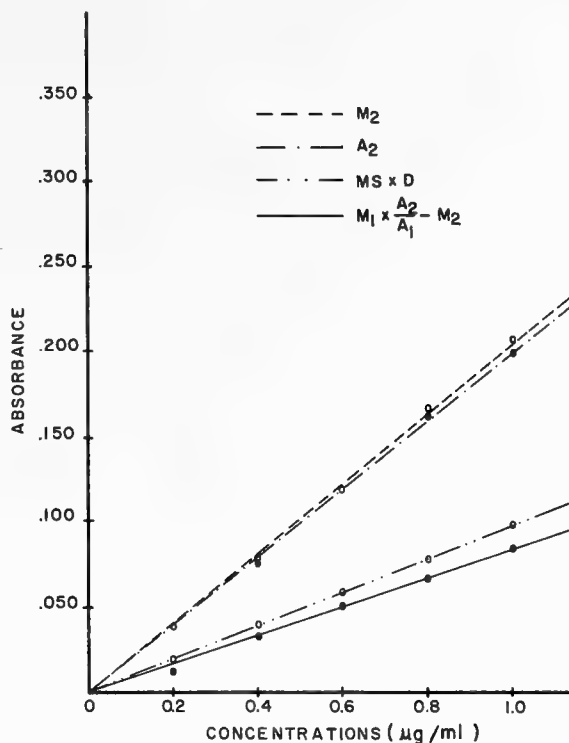


Figure 3.--Absorbance curves at 545 nm for: (A_2) standards of m-aminobenzoic acid extracted with carbon tetrachloride; (M_2) standards of MS-222 and m-aminobenzoic acid mixed and extracted with carbon tetrachloride; ($M_1 \times \frac{A_2}{A_1} - M_2$) the calculated absorbance of MS-222 after extracting the acid from the mixed standards; and (MS X D) standard curve of MS-222 multiplied by the known dilution factor resulting from the extraction of the acid.

When a fresh standard of MS-222 is diazotized and extracted with basic carbon tetrachloride, the aqueous fraction has zero absorbance. Thus, the amount of MS-222 present in a sample has to be calculated from the information obtained by extracting a 1- μ g/ml standard of acid and the absorbance values of a mixed 1- μ g/ml standard of MS-222 and m-aminobenzoic acid before and after extraction.

The amount of recoverable MS-222 and m-aminobenzoic acid was determined by spiking samples of largemouth bass (Micropterus salmoides) and liver tissue with 1 to 10 μ g/g amounts of the two compounds (table 1). Recovery studies were done only on muscle and liver tissues because interfering substances

Table 1.--Recovery of MS-222 and m-aminobenzoic acid spiked into 1-g samples of largemouth bass muscle and liver tissue as determined by the colorimetric method and confirmed by the thin-layer chromatographic method

Tissue	Number of analyses	Concentration added (μ g)		Concentration recovered (μ g)		Percent recovery		Confirmed by TLC	
		MS-222	Acid	MS-222	Acid	MS-222	Acid	MS-222	Acid
Muscle	3	1.0	0.0	0.827	--	82.7	--	Yes	No
	3	5.0	0.0	4.740	--	94.8	--	Yes	No
	3	7.5	0.0	6.750	--	90.0	--	Yes	No
	3	10.0	0.0	10.680	--	106.8	--	Yes	No
	3	0.0	1.0	--	0.860	--	86.0	No	Yes
	3	0.0	5.0	--	4.970	--	99.6	No	Yes
	3	0.0	7.5	--	7.470	--	99.6	No	Yes
	3	0.0	10.0	--	9.950	--	99.5	No	Yes
	3	1.0	1.0	0.870	0.910	87.0	91.0	Yes	Yes
	3	2.5	7.5	2.730	7.340	109.2	97.9	Yes	Yes
	3	5.0	5.0	4.790	4.870	95.8	97.4	Yes	Yes
	3	7.5	2.5	7.720	2.180	102.9	87.2	Yes	Yes
Liver	1	5.0	0.0	4.880	--	97.6	--	Yes	No
	1	10.0	0.0	10.230	--	102.3	--	Yes	No
	1	0.0	5.0	--	4.780	--	95.6	No	Yes
	1	0.0	10.0	--	10.500	--	105.0	No	Yes
	1	2.5	7.5	2.650	8.770	106.0	116.9	Yes	Yes
	1	7.5	2.5	8.270	2.100	110.3	84.0	Yes	Yes

are most prevalent in liver and least prevalent in muscle tissue. Recoveries ranged from 82.7 to 110.3 percent for MS-222 and from 84.0 to 116.9 percent for m-aminobenzoic acid.

Diazotization of compounds mentioned in this study was accomplished by modification of the Bratton and Marshall (1939) procedure described by Walker and Schoettger (1967). The reaction of primary aromatic amines with nitrous acid yields a diazonium salt, which will couple with certain aromatic amines to yield strongly colored azo compounds. Naturally occurring primary aromatic amines in certain fish tissues, especially liver, react with the Bratton-Marshall reagents and cause serious quantitative errors in the determination of MS-222 and m-aminobenzoic acid residues. Thus, background levels of these amines are determined in control samples

and subtracted from levels obtained in treated samples. This is an accepted procedure in any analytical method by spectrophotometry.

Another standard procedure should be a confirmatory method of analysis. The thin-layer chromatographic method described previously will separate azo dyestuffs of naturally occurring primary aromatic amines from MS-222 and m-aminobenzoic acid. The following R_f values were obtained: 0.83 for the azo dyestuff of MS-222, 0.70 for m-aminophenol, 0.34 for sulfanilic acid, 0.26 for p-aminobenzoic acid, 0.18 for m-aminobenzoic acid, and 0.00 for liver extract. The comparison of R_f values must be made on the same thin-layer plate since these values may vary between determinations.

The minimum amount of azo dyestuff from a MS-222 standard that can be visualized on a

thin-layer plate was found to be 5 ng. The maximum amount of azo dyestuff from muscle extract that can be spotted on a thin-layer plate is 100 μ l, which is equivalent to 25 mg of tissue. Thus, the sensitivity of the method is 0.2 ng/mg. Quantitative estimations of residues in samples can be accomplished by spotting a series of MS-222 and *m*-aminobenzoic acid standards in the range of 5 to 25 ng amounts on the same plate along with the samples.

DISCUSSION

The analytical and confirmatory methods described here will effectively determine and positively confirm which metabolites of MS-222 are present. Different species of fish seem to eliminate residues of MS-222 by various means of excretion and deactivation,

which in turn will influence the amount of metabolites present in the muscle (table 2).

Excellent recoveries of MS-222 and *m*-aminobenzoic acid were obtained from spiked samples of muscle tissue. Recoveries were higher from spiked liver samples because of inconsistent background interference from naturally occurring primary aromatic amines, which amounted to a mean of 89 percent transmittance in the control liver samples after extraction. By thin-layer chromatography, the control liver samples contained no spot with the same R_f value as MS-222 or *m*-aminobenzoic acid.

The accuracy of the analytical method is governed by the tissue being analyzed and by the techniques of extraction and dilution. The dilution factor attributed to extraction

Table 2.--Average concentrations of MS-222, *m*-aminobenzoic acid, and their N-acetyl derivatives in muscle tissue of four species of fish after a 15-minute exposure to a 100-mg/l aqueous solution of MS-222 at 18° C.

	Number of fish	Mean concentration of residues by colorimetric method				Free residues confirmed by TLC	
		Free MS-222 (μ g/g)	Acetylated MS-222 (μ g/g)	Free acid (μ g/g)	Acetylated acid (μ g/g)	MS-222	Acid
Channel catfish (<i>Ictalurus punctatus</i>)	5	67.9 ¹ (0.7)	4.5	3.4(0.5)	1.7	Yes	Yes
Striped bass (<i>Morone saxatilis</i>)	5	23.0(0.5)	0.0	14.7(1.2)	3.3	Yes	Yes
Bluegill (<i>Lepomis macrochirus</i>)	5	23.8(1.0)	0.6	0.0(0.0)	0.0	Yes	No
Largemouth bass (<i>Micropterus salmoides</i>)	5	34.4(1.9)	0.0	1.4(0.0)	0.7	Yes	Yes

¹ Residues after 6 hours recovery in fresh water at 18° C; not analyzed by TLC.

($A_2/A_1 = 0.725$) as determined by the extraction of a 1- $\mu\text{g/ml}$ standard of m-aminobenzoic acid was quite consistent. Particular care must be taken to remove all gas bubbles that form on the sides of the cuvettes. Generally, these bubbles will float to the top when the sides of the cuvette are tapped lightly with a soft object. The maximum absorbance for the azo dyestuff of m-aminobenzoic acid is identical to that of MS-222, which is 545 nm.

CONCLUSIONS

1. The colorimetric method presented will effectively determine MS-222 and m-aminobenzoic acid residues in fish tissues.
2. Recovery of the compounds from spiked samples of muscle and liver tissues ranged from 82.7 to 110.3 for MS-222 and from 84.0 to 116.9 percent for m-aminobenzoic acid.
3. The thin-layer chromatographic method presented will positively identify and separate MS-222 and m-aminobenzoic acid residues in fish tissues.
4. The R_f values for the azo dyestuff of MS-222 and m-aminobenzoic acid are near 0.83 and 0.18, respectively.

5. Quantitative estimation and identification by the thin-layer chromatographic method were possible from samples spiked with as little as 1 $\mu\text{g/g}$ of MS-222 or m-aminobenzoic acid.

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52. Residues of MS-222, Benzocaine, and Their Metabolites in Striped Bass Following Anesthesia

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United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

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RESIDUES OF MS-222, BENZOCAINE, AND THEIR METABOLITES IN STRIPED BASS FOLLOWING ANESTHESIA

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ABSTRACT.--Striped bass (*Morone saxatilis*) anesthetized in a 100-mg/l solution of MS-222 at 17.5°C contained an average of 57.9 µg/g of MS-222 and 23.3 µg/g of *m*-aminobenzoic acid residues in the muscle tissue immediately after a 30-minute exposure to the drug (0-hour withdrawal samples). After this tissue was homogenized and stored in a refrigerator for 1 week at 1.7°C, residues were 100 percent *m*-aminobenzoic acid. Fish anesthetized with benzocaine and treated in like manner still contained residues of benzocaine and a small amount of *p*-aminobenzoic acid (3.4 percent) after storage. The ester and acid residues of both anesthetics decreased steadily with length of recovery time. The residues were measured by a modified Bratton-Marshall colorimetric method and confirmed by thin-layer chromatography.

INTRODUCTION

MS-222 (methanesulfonate of *meta*-aminobenzoic acid ethyl ester) is an effective and widely used fish anesthetic (Schoettger, 1967; Schoettger and Julin, 1967; Schoettger, Walker, Marking, and Julin, 1967). Residues of this drug occur in various tissues of anesthetized fish, but decline to background levels within 9-24 hours after fish are placed into fresh water (Walker and Schoettger, 1967a). Other than the fact that residues do disappear, little is known about the metabolic fate of MS-222. In striped bass muscle tissue analyzed by the modified method of Walker and Schoettger (1967b), MS-222 residues were found in 0-hour through 8-hour withdrawal samples. When these same samples were analyzed by the thin-layer chromatographic method (Allen, Luhning, and Harman, 1970), only the 0-hour withdrawal samples contained MS-222 residues. These results implied that the former method did not distinguish between MS-222 and some metabolite. The metabolite was identified as *m*-aminobenzoic acid and was analyzed by the method of Luhning (1973).

The objective of this study was to determine the amount of MS-222 and *m*-aminobenzoic acid residue in muscle tissue of striped bass anesthetized with MS-222. Analyses of benzocaine, a structural analogue of MS-222, and *p*-aminobenzoic acid residues in muscle tissue of striped bass, bluegill, and largemouth bass anesthetized with benzocaine were included for comparative purposes.

METHODS AND MATERIALS

Fish

Striped bass (*Morone saxatilis*), ranging from 12.7 to 20.3 cm long, were obtained from the National Fish Hatcheries at Edenton, N.C., and Welaka, Fla. Bluegill (*Lepomis macrochirus*), ranging from 16.5 to 21.6 cm long, and largemouth bass (*Micropterus salmoides*), ranging from 16.5 to 25.4 cm long, were obtained from the National Fish Hatchery at Marion, Ala. All fish were held in limed, spring water at the Warm Springs Laboratory and were maintained according to the methods of Hunn, Schoettger, and Whealdon (1968).

Anesthetization of fish

I used technical-grade (99.4-percent) MS-222, the methane sulfonate of m-aminobenzoic acid ethyl ester, marketed for experimental purposes by Ayerst Laboratories, Inc., New York, N.Y., under the trade name of FIN-QUEL(R). The m- and p-aminobenzoic acid and p-aminobenzoic acid ethyl ester were purchased from Eastman Kodak Company.¹

Striped bass, bluegill, and largemouth bass were anesthetized for 30 minutes in a 100-mg/l aqueous solution of MS-222 buffered to pH 7.0 with sodium bicarbonate. Also, these three species of fish were anesthetized for 15 minutes in a 63.216-mg/l aqueous solution of benzocaine (63.216 mg/l para-aminobenzoic acid ethyl ester is equal on a mole basis to 100 mg/l methanesulfonate salt of meta-aminobenzoic acid ethyl ester) buffered to pH 6.5 with sodium bicarbonate. All fish were exposed to 75 l of anesthetic solution at a temperature of $18 \pm 0.5^{\circ}$ C.

Benzocaine is only slightly soluble in water. To obtain the concentration of anesthetic equivalent to that of MS-222, the benzocaine was dissolved in ethanol containing a small amount of methanesulfonic acid. Thus, 4.7412 g of benzocaine dissolved in 50 ml of ethanol were added to 75 l of limed, spring water to obtain a concentration of anesthetic equivalent to 100 mg/l of MS-222.

After the specified exposure time, the fish were withdrawn from the anesthetic solution, and placed in fresh, flowing water for recovery. This action marked the beginning of withdrawal time. Fish killed immediately after withdrawal from the anesthetic solution were labeled as 0-hour withdrawal samples. Five fish were used for residue analyses at each withdrawal interval.

Analyses of samples

The head, scales, fins, and viscera were removed from each fish, and the remaining

tissues were homogenized in a Waring blender. One-gram samples of these homogenates were analyzed for residues of MS-222, benzocaine, and their acid metabolites on the same day the fish were killed. The remainder of each homogenate was stored in a refrigerator at 1.7° C, and a sample was analyzed 1 week later. One-gram samples of liver and 1 ml of blood from some fish also were analyzed on the same day the fish were killed.

Samples of homogenized tissue were initially analyzed by the colorimetric method described by Walker and Schoettger (1967b). In addition to this method, the azo dyestuff of each sample was partitioned with basic carbon tetrachloride, centrifuged, and the aqueous layer separated, and made acidic (Luhning, 1973). This aqueous fraction contained only the azo dyestuff of the acid (m- or p-aminobenzoic acid).

Standard solutions of mixed ester and acid at a concentration of 1 μ g/ml each, also were partitioned. The amounts of ester and acid present in the samples were calculated on a ratio basis with standards of the ester and acid partitioned in the same manner as the samples.

RESULTS

MS-222 treated fish

Twenty striped bass were anesthetized for 30 minutes in a 100-mg/l solution of MS-222 at 17.5° C. Deep anesthesia in striped bass occurred about the same time as it did in the largest specimens among the largemouth bass. No mortalities occurred with bluegill or largemouth bass, whereas one striped bass died during the 24-hour recovery period.

Striped bass rapidly hydrolyzed the m-aminobenzoic acid ethyl ester to m-aminobenzoic acid during exposure and withdrawal (table 1). During the first 4 hours of recovery, residues of m-aminobenzoic acid dissipated from the muscle tissue at a slower rate than did the MS-222 residues. For comparison, five bluegill and five largemouth bass anesthetized and analyzed in the same way as the striped bass contained a small percentage of total residue as acid at the 1-hour withdrawal interval.

¹Reference to a company or product does not imply recommendation to the exclusion of others that may be suitable.

Table 1.--Average concentrations of MS-222 and m-aminobenzoic acid residues in muscle tissue of five striped bass, bluegill, and largemouth bass following deep anesthesia in a 100-mg/l aqueous solution of MS-222 buffered to pH 7.0 at 17.5° C

Species	Exposure time (minutes)	Withdrawal time (hours)	Free MS-222 residues (μg/g)		Free <u>m</u> -aminobenzoic acid residues (μg/g)	
			Mean ± $s \frac{1}{x}$	Range	Mean ± $s \frac{1}{x}$	Range
Striped bass ²	0	0	0.0	--	0.3 ± 0.09	0.0- 0.5
	30	0	57.9 ± 5.59	36.1-66.5	23.3 ± 2.63	15.3-30.6
		1	6.5 ± 1.06	2.6- 8.9	16.5 ± 1.94	11.1-21.1
		4	0.8 ± 0.23	0.2- 1.5	7.5 ± 0.79	5.7-10.0
		24	³ 0.5 ± 0.16	0.2- 0.8	0.2 ± 0.06	0.0- 0.2
Bluegill ⁴	30	1	4.8 ± 0.27	3.7- 5.2	0.5 ± 0.13	0.2- 0.9
Largemouth bass ⁵	30	1	8.1 ± 0.61	5.8- 9.5	0.3 ± 0.10	0.0- 0.6

¹ Standard error of the mean for five fish.

² Average length of 15.2 cm and average weight of 46.9 g.

³ Mean of four fish.

⁴ Average length of 20.1 cm and average weight of 179.0 g.

⁵ Average length of 20.3 cm and average weight of 154.0 g.

A limited number of striped bass were available to test the rate of hydrolysis during a 50-minute exposure to a 100-mg/l aqueous solution of MS-222 and at intervals during recovery from the drug in fresh, flowing water (table 2). The amount of residue accumulated after 30 minutes of exposure and no recovery time was 44.6 μg/g, of which 49.6 percent was free MS-222. This amount is not in agreement with the data found in tables 1 and 4. This discernible difference could have been due to fish size, number of fish analyzed, temperature difference, source of fish difference, or experimental error.

The added information gained from this test was the fact that m-aminobenzoic acid residues continued to increase during 50 minutes of anesthesia and MS-222 residues started to decrease after 30 minutes of

anesthesia (fig. 1). Analyses of acetylated MS-222 and m-aminobenzoic acid also were performed on these samples, and only a small amount of the residue was found to be acetylated, mostly as m-acetylaminobenzoic acid (table 2).

Hydrolysis of MS-222 also occurred in homogenized striped bass tissue during storage (table 3). Samples of homogenized muscle tissue from striped bass, bluegill, and largemouth bass were previously analyzed for MS-222 and m-aminobenzoic acid residues (table 1). Additional samples were analyzed after storage for 1 week at 1.7° C. The 0-hour withdrawal samples of striped bass contained an average of 51.6 μg/g acid residues, but no residues of MS-222 could be detected at the end of the storage period. A reduction in the MS-222 concentration occurred during storage of

Table 2.--Residues of MS-222, m-aminobenzoic acid and their N-acetyl derivatives in muscle tissue of striped bass during different times of exposure to and recovery from a 100-mg/l aqueous solution of MS-222 buffered to pH 7.0 at 18.5° C

Exposure time (minutes)	Withdrawal time (minutes)	Average free ¹ residues (μg/g)		Average acetylated ² residues (μg/g)		Total residues (μg/g)	Percent free MS-222
		MS-222	Acid	MS-222	Acid		
15	0	19.0	11.9	0.0	3.3	34.2	55.6
30	0	22.1	19.4	0.0	3.1	44.6	49.6
50	0	15.8	24.3	0.0	2.5	42.6	37.1
30	15	13.2	17.8	0.0	2.2	33.2	39.8
30	30	12.3	11.6	0.0	2.0	25.9	47.5
30	45	7.8	11.6	0.5	1.5	21.4	36.4
30	60	³ 5.6	9.4	0.0	1.6	16.6	33.7

¹ Average of two fish having an average length and weight of 17.8 cm and 67.6 g., respectively.

² Obtained by subtracting the free residue and background concentration of control (untreated) fish from the amount measured in the samples after acid hydrolysis and extraction.

³ Average of two analyses from one fish.

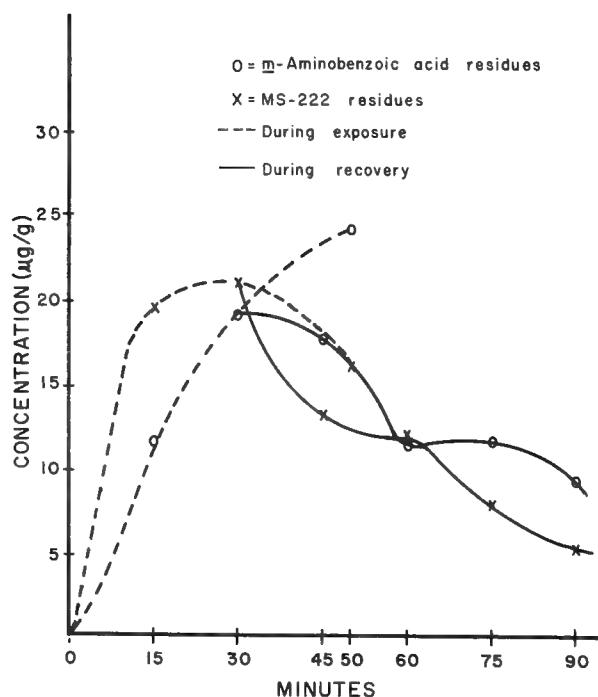


Figure 1.--Uptake and hydrolysis of MS-222 residues in striped bass muscle tissue during different lengths of exposure to a 100-mg/l solution of MS-222 buffered to pH 7.0 at 18.5° C, and elimination of MS-222 and m-aminobenzoic acid residues during 1 hour of recovery time in fresh water.

Table 3.--Residues of free MS-222, benzocaine, and their acid metabolites in homogenized muscle tissue of three species of treated fish stored for 1 week at $1.7^{\circ} \pm 0.2^{\circ} \text{C}$

Species	Withdrawal time (hours)	MS-222 treated ¹		Benzocaine treated ²	
		MS-222 ($\mu\text{g/g}$)	<u>m</u> -aminobenzoic acid ($\mu\text{g/g}$)	Benzocaine ($\mu\text{g/g}$)	<u>p</u> -aminobenzoic acid ($\mu\text{g/g}$)
		Mean \pm s $\frac{3}{x}$	Mean \pm s $\frac{3}{x}$	Mean \pm s $\frac{3}{x}$	Mean \pm s $\frac{3}{x}$
Striped bass	0	0.0	51.6 ± 5.73	25.8 ± 1.83	0.9 ± 0.10
	1	0.1 ± 0.07	16.5 ± 2.44	3.3 ± 0.19	0.2 ± 0.10
	4	0.5 ± 0.17	5.8 ± 0.81	0.6 ± 0.05	0.1 ± 0.04
Bluegill	1	3.8 ± 0.61	0.6 ± 0.08	1.5 ± 0.50	0.0
		⁴ (0.0)	(0.6)	(0.0)	(0.6)
Largemouth bass	1	5.0 ± 0.99	0.4 ± 0.02	2.1 ± 0.32	0.06 ± 0.04
		(0.0)	(0.6)	(0.0)	(0.4)

¹ Anesthetized for 30 minutes in a 100-mg/l solution of MS-222 buffered to pH 7.0 at 17.5°C .

² Anesthetized for 15 minutes in a 63.216-mg/l solution of benzocaine (equivalent to 100-mg/l of MS-222) buffered to pH 6.5 at 17.8°C .

³ Standard error of the mean for five samples.

⁴ Acetylated fraction obtained by subtracting the free and background concentrations of control (untreated) fish from the amount measured in the samples after acid hydrolysis and extraction.

homogenized muscle tissue from bluegill and largemouth bass, but no significant change occurred in the acid concentration (tables 1 and 3).

In another test using 0-hour withdrawal samples of striped bass tissue, the temperature at which samples were stored greatly influenced the rate at which MS-222 residues were hydrolyzed to the acid (tables 4 and 5). Homogenized tissue stored at -12.2°C for 3 weeks contained an average of $21.7 \mu\text{g/g}$ MS-222 residues. However, after this tissue was stored for an additional 8 days at 4.4°C , it contained no residues of MS-222.

Residues of MS-222 and m-aminobenzoic acid were confirmed in all the 0- and 1-hour withdrawal samples of striped bass by the thin-layer chromatographic method described by Luhning (1973).

Benzocaine treated fish

Striped bass, bluegill, and largemouth bass were anesthetized in a 63.216-mg/l aqueous solution of benzocaine for 15 minutes at 17.8°C . Deep anesthesia occurred in about the same time as it did when MS-222 was used. No mortalities of largemouth bass or bluegill occurred before the withdrawal time of 1 hour, but one striped bass died during the 24-hour recovery period.

Benzocaine residues comprised from 93.5 to 100 percent of the total residues found in these three species of fish (table 6). The mean residues of p-aminobenzoic acid in striped bass and largemouth bass were about the same concentration at the 1-hour withdrawal interval, whereas residues of m-aminobenzoic acid differed greatly between these two species at the same withdrawal interval (tables 1 and 6).

Table 4.--Residues of MS-222 and m-aminobenzoic acid in 0-hour withdrawal samples of striped bass muscle tissue during storage at -12.2° C

Storage time in weeks	Average $\mu\text{g/g}$ free residues ¹				Percent MS-222
	MS-222		<u>m</u> -aminobenzoic acid		
	Mean	Range	Mean	Range	
0	35.4	30.4-44.9	6.9	5.6- 8.0	83.7
1	28.4	22.3-36.8	11.2	10.0-12.2	71.7
2	24.5	15.9-31.9	12.1	10.0-14.0	66.9
3	21.7	12.3-31.9	12.7	10.0-15.3	62.8
4	18.2	9.9-26.4	13.2	11.7-16.0	58.0
5	16.3	8.1-20.9	14.3	12.6-16.9	53.3
6	15.3	6.9-20.3	15.7	14.1-17.9	49.4
8	15.0	6.8-20.8	16.0	14.1-17.9	48.4

¹ Average of three fish, which were exposed for 30 minutes in a 100-mg/l aqueous solution of MS-222 at 20° C.

Table 5.--Residues of MS-222 and m-aminobenzoic acid in 0-hour withdrawal samples of striped bass muscle tissue kept at -12.2° C for 3 weeks, then analyzed during storage at 4.4° C

Storage time in days	Average $\mu\text{g/g}$ free residues ¹				Percent MS-222
	MS-222		<u>m</u> -aminobenzoic acid		
	Mean	Range	Mean	Range	
0	21.7	12.3-31.9	12.7	10.0-15.3	62.8
1	13.2	5.8-19.6	17.0	16.3-17.6	43.7
3	5.2	0.4- 8.2	20.7	19.7-22.5	20.1
6	0.3	0.0- 0.9	23.7	21.8-26.4	1.3
8	0.0	--	24.9	22.5-28.2	0.0

¹ Average of three fish, which were exposed for 30 minutes in a 100-mg/l aqueous solution of MS-222 at 20° C.

Table 6.--Average concentrations of benzocaine and *p*-aminobenzoic acid residues in muscle tissue of five striped bass, bluegill, and largemouth bass following deep anesthesia in a 63.216-mg/l aqueous solution of benzocaine buffered to pH 6.5 at 17.8° C

Species	Exposure time (minutes)	Withdrawal time (hours)	Free benzocaine residues (μg/g)		Free <i>p</i> -aminobenzoic acid residues (μg/g)	
			Mean ± $\frac{s}{\sqrt{x}}$	Range	Mean ± $\frac{s}{\sqrt{x}}$	Range
Striped bass ²	0	0	0.0	--	0.3 ± 0.09	0.0-0.5
	15	0	37.9 ± 2.43	28.6-41.4	1.5 ± 0.46	0.8-3.3
		1	7.3 ± 0.99	3.4- 8.8	0.2 ± 0.07	0.0-0.4
		4	0.7 ± 0.21	0.1- 1.2	0.1 ± 0.04	0.0-0.2
		24	0.2 ± 0.07	0.0- 0.5	0.0	--
Bluegill ³	15	1	1.9 ± 0.51	0.9- 3.8	0.0	--
Largemouth bass ⁴	15	1	2.9 ± 0.57	2.0- 4.8	0.2 ± 0.00	--

¹ Standard error of the mean for five fish.

² Average length of 16.0 cm and average weight of 54.5 g.

³ Average length of 18.0 cm and average weight of 154.0 g.

⁴ Average length of 19.1 cm and average weight of 106.8 g.

Both the ester and acid residues decreased during storage in a refrigerator at $1.70 \pm 0.20^\circ \text{C}$ for 1 week (table 3). This decrease was due mainly to acetylation of the free amine. Residues of benzocaine and *p*-aminobenzoic acid were confirmed in all of the 0-hour withdrawal samples of striped bass by thin-layer chromatography.

DISCUSSION

Striped bass muscle tissue contains an esterase or a substance capable of cleaving the ester of MS-222. Muscle tissue of striped bass spiked with MS-222 and analyzed 1 week later contained both MS-222 and *m*-aminobenzoic acid. It is not known whether the muscle tissue is completely responsible for the ester cleavage while the fish is still alive. Preliminary investigations showed that *m*-aminobenzoic acid residues amounted to 27.1 percent of the total free residues in striped bass muscle tissue, 20.6 percent in blood, and 15.8 percent in liver after a 30-minute exposure in a 100-mg/l solution of MS-222. In the 1-hour withdrawal samples, muscle tissue had the

lowest concentration of free acid when compared to blood and liver. No residues of acetylated MS-222 were detected in the 0-hour withdrawal samples of blood, liver, and muscle. In the 0-hour withdrawal samples, residues of *m*-acetylaminobenzoic acid were highest in the blood (22.5 μg/g) and lowest in the muscle (1.7 μg/g).

A limited amount of information is available on the acid metabolite of MS-222, *m*-aminobenzoic acid, in freshwater fishes. On the other hand, a considerable amount of data is available on the *N*-acetyl derivative of MS-222, but no differentiation is made between the acetylated ester and the acid (Schoettger, Walker, Marking, and Julin, 1967; Hunn, Schoettger, and Willford, 1968). Presumably MS-222 and acetylated MS-222 are eliminated by the gills during recovery, whereas *m*-aminobenzoic acid and *m*-acetylaminobenzoic acid are excreted renally (Hunn, 1970). Investigations by Maren, Broder, and Stenger (1968) on the metabolism of ethyl-*m*-aminobenzoate (MS-222) in the dogfish shark (*Squalus acanthias*), revealed that 2 hours after dogfish sharks were

injected with MS-222, the predominant metabolites in the urine were m-aminobenzoic acid and m-acetylaminobenzoic acid. Hunn, Schoettger, and Willford (1968) reported that acetylated MS-222 was found in much higher concentrations in the urine than in the blood of rainbow trout (*Salmo gairdneri*). No analyses were done on urine of striped bass.

In this study, residues of MS-222 and m-aminobenzoic acid steadily decreased with length of recovery time. On a percentage basis of all residues determined, m-aminobenzoic acid residues increased with recovery time in striped bass muscle tissue, but not in muscle tissue of bluegill and largemouth bass. Residue concentrations of m-aminobenzoic acid increased with an increase in sample storage time only in striped bass muscle tissue, whereas residue concentrations of p-aminobenzoic acid remained relatively low in fresh and stored muscle tissue of all fish analyzed. The decrease in the concentration of free benzocaine residues which occurred in all stored fish samples was due mainly to acetylation of the free amine. Thus, samples should be analyzed as soon as possible to obtain data pertinent to each withdrawal interval.

Residues of acetylated MS-222 (m-acetylaminobenzoic acid ethyl ester) cannot be determined accurately by the acetylation test of Walker and Schoettger (1967b). In this test the acetylated fraction is obtained by subtracting the free MS-222 and background concentrations from the total aromatic amines measured in the sample after acid hydrolysis. When the ester and acid fractions are separated before and after acid hydrolysis, the MS-222 fraction obtained after hydrolysis is generally less than the amount obtained before hydrolysis. This indicates that some free MS-222 is converted to free m-aminobenzoic acid during the acid hydrolysis. Thus, the esterified compounds should be quantified before and after acid hydrolysis, to determine the amount of free MS-222 and acetylated MS-222 that might be converted to free m-aminobenzoic acid during hydrolysis.

CONCLUSIONS

1. Among the species of fish analyzed to date, only the striped bass effectively hydrolyzes

the ester of MS-222 to m-aminobenzoic acid in vivo.

2. Hydrolysis of MS-222 also occurs in frozen, homogenized muscle tissue from treated striped bass.
3. A reduction in the concentration of MS-222 residues after 1 week storage at 1.7° C in bluegill and largemouth bass muscle tissue was due mainly to the acetylation of the free amine.
4. Analysis of striped bass muscle tissue for MS-222 residues must be done soon after the fish are killed to determine the amount of parent drug rather than its acid metabolite, because hydrolysis occurs during storage.

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INVESTIGATIONS IN FISH CONTROL

- 53. Toxicity of Mixtures of Quinaldine Sulfate and MS-222 to Fish**
- 54. The Efficacy of Quinaldine Sulfate:MS-222 Mixtures for the Anesthetization of Freshwater Fish**
- 55. Residues of Quinaldine and MS -222 in Fish Following Anesthesia with Mixtures of Quinaldine Sulfate:MS-222**



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50. Residue of Quinaldine in Ten Species of Fish Following Anesthesia with Quinaldine Sulfate, by Joe B. Sills, John L. Allen, Paul D. Harman, and Charles W. Luhning. 1973. 9 p.

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FOREWORD

All chemicals, including anesthetics, which are to be used on food or game fishes must be approved and registered by the Food and Drug Administration. Research to support petitions for registration of such compounds is an integral part of the program of the Fish Control Laboratories. Studies involving anesthetics have centered on quinaldine and tricaine methanesulfonate (MS-222, Finquel^(R)), the two most commonly used anesthetics for fish.

While each compound is effective in itself, studies have shown that when MS-222 and quinaldine are used together, desired anesthesia is achieved without undesirable side effects noted when the compounds are used singly.

Registration-oriented research on MS-222 was reported in Investigations in Fish Control (IFC), numbers 12-17. The development of a water-soluble salt of quinaldine and related studies to support a petition for its registration are found in IFC, numbers 47-50.

The papers which follow are concerned with research on the toxicity, efficacy, and residues associated with the use of mixtures of quinaldine sulfate and MS-222 as an anesthetic for selected coldwater and warmwater fishes. The data presented will be used to support a petition for registration to permit the use of such mixtures on fish.

Fred P. Meyer, Director
Fish Control Laboratories

53. Toxicity of Mixtures of Quinaldine Sulfate and MS-222 to Fish

By Verdel K. Dawson and Leif L. Marking



United States Department of the Interior
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TOXICITY OF MIXTURES OF QUINALDINE SULFATE AND MS-222 TO FISH

By Verdel K. Dawson and Leif L. Marking
Fish Control Laboratory, La Crosse, Wisconsin

ABSTRACT.--The acute toxicities of mixtures of two fish anesthetics (quinaldine sulfate and MS-222) to coho salmon, rainbow trout, brown trout, brook trout, lake trout, carp, channel catfish, bluegill, and largemouth bass of various sizes were determined in 15-, 30-, and 60-minute and 24-, 48-, and 96-hour static toxicity tests. The effects of various temperatures, water hardnesses, and pH's on the mixture's toxicity were evaluated. The 96-hour LC50's of QdSO₄:MS-222 in ratios of 1:4 ranged from 4.23:16.9 mg/l for lake trout to 8.63:34.5 mg/l for carp in standard reconstituted water at 12° C. Temperature changes had little influence on the effect of the drugs. In very soft water, solutions of the combination are acidic and considerably less toxic than in harder water. The toxicity of the mixture decreases with decreasing pH, especially below pH 6.5. Safety indices (lethal concentration/effective concentration) indicate that the safety margin is greater at shorter exposures.

INTRODUCTION

An anesthetic is an important tool for handling fish during operations such as artificial spawning, marking, weighing, measuring, transporting, and others. MS-222 has been shown to be an effective anesthetic for fish and other coldblooded organisms. The action of MS-222 is characterized by rapid and deep anesthetization, but concentrations that render fish immobile are not tolerated for extended periods (McFarland, 1959; Bové, n.d.; Schoettger and Julin, 1967). Bové (n.d.) identified MS-222 as the methanesulphonate of meta-aminobenzoic acid ethyl ester. The compound is a fine, white crystalline powder which is soluble to 11 percent in water and forms a clear, colorless, acidic, and relatively stable solution.

The anesthetic effect of quinaldine (2-methylquinoline) on fish was first reported by Muench (1958). Schoettger and Julin (1969) further investigated the use of quinaldine as an anesthetic for several species of hatchery-reared fish under a variety of temperature and water quality conditions. The action of quinaldine in fish is characterized by long, safe exposure times, but it does not entirely

block reflex movements. Quinaldine occurs in coal tar and is made from aniline, acet-aldehyde, and hydrochloric acid. It is a colorless, oily liquid that turns reddish-brown upon exposure to air. Quinaldine is soluble in alcohol, ether, chloroform, and acetone but is insoluble in water (Stecker, 1968).

Schoettger and Steucke (1970) reported mixtures of quinaldine and MS-222 to be synergic for anesthetizing fish. The combination of anesthetics exhibits the rapid sedation and lack of reflex response typical of MS-222 and the long, safe exposure time typical of quinaldine.

Amendments to the Federal Food, Drug, and Cosmetic Act require that chemicals used on fish be registered for their specific uses (Lennon, 1967). The registration of MS-222 has been supported by information on its toxicity or maximum safe exposure to fish (Marking, 1967) and the persistence of residues in fish tissues (Walker and Schoettger, 1967).

Recently, Allen and Sills (1973) synthesized quinaldine sulfate (QdSO₄), a salt of quinaldine, which is water soluble and has a less pungent odor than quinaldine. Gilderhus, et al. (1973)

evaluated the efficacy of QdSO_4 to 15 species of fish and found it to be as effective, on the basis of active ingredient, as that of quinaldine. The toxicity of QdSO_4 to fish under a variety of conditions was determined by Marking and Dawson (1973). Sills, et al. (1973) measured QdSO_4 residues in 10 species of fish.

Because QdSO_4 is more convenient to use than quinaldine, tests of mixtures of QdSO_4 and MS-222 were devised. The purpose of this investigation was to define concentrations of three ratios of the combination which are toxic to various species and sizes of freshwater fish at selected exposure periods in water at three temperatures, four water hardnesses, and four pH's. In addition, the safety must be determined for use pattern concentrations and exposures.

METHODS AND MATERIALS

The QdSO_4 (quinaldine sulfate) was synthesized at the Southeastern Fish Control Laboratory, Warm Springs, Ga. The MS-222 (methane sulfonate of meta-aminobenzoic acid ethyl ester) was Finquel^(R), marketed by Ayerst Laboratories, Inc.

Static toxicity tests of mixtures of QdSO_4 and MS-222 were conducted with 3- to 6-cm fish in glass jars containing 15 l of water according to the methods of Lennon and Walker (1964). Larger fish were exposed to the anesthetics in 45-l polyethylene tanks. The two drugs were tested for toxicity against coho salmon (Oncorhynchus kisutch), rainbow trout (Salmo gairdneri), brown trout (Salmo trutta), brook trout (Salvelinus fontinalis), lake trout (Salvelinus namaycush), carp (Cyprinus carpio), channel catfish (Ictalurus punctatus), bluegill (Lepomis macrochirus), and largemouth bass (Micropterus salmoides). The fish were obtained from fish hatcheries, maintained under a fish culturist's care (Hunn, et al., 1968), acclimated to the test water before the chemical was added, and incinerated after death. Ten fish were exposed to each concentration of the anesthetics, and mortalities were recorded periodically the

first day and daily thereafter during the 96-hour tests.

The hardness of the test water was altered by adding selected amounts of reconstituting salts to deionized water, and the pH in certain tests (ranging from 6.5 to 9.5) was adjusted and maintained with chemical buffers (Marking and Dawson, 1973). Temperatures of 7⁰, 12⁰, and 17⁰ C were controlled by water baths.

Stock solutions of the anesthetics dissolved in water were added to the bioassays to obtain the desired concentrations. QdSO_4 and MS-222 were tested in a ratio of 1:4 against the nine available species, while additional ratios of 1:6 and 1:2 were tested against representative coldwater and warmwater species.

The mortality data were analyzed according to the method of Litchfield and Wilcoxon (1949) to determine LC50's (concentration causing 50 percent mortality), variations, slope functions, and 95-percent confidence intervals.

Fingerling rainbow trout (1.1 g) were exposed to mixtures of QdSO_4 and MS-222 (1:2) to determine safety indices (Marking, 1967). A safety index refers to the margin between efficacy and mortality and is expressed by the quotient of a lethal concentration (LC50) and an effective concentration (EC50). The EC50 defines the concentration of drugs which produces total loss of equilibrium (stage 2) in half the organisms (Schoettger and Julin, 1967). The maximum safety index (LC1/EC99) is lower than the safety index and is biased in favor of greater safety.

The toxicity of mixtures of the anesthetics was defined by an additive index¹ developed at the Fish Control Laboratory. The index expresses the toxicity quantitatively with zero indicating strictly additive toxicity. Negative values indicate less than additive toxicity and positive values indicate greater than additive toxicity.

¹Leif L. Marking and Verdel K. Dawson. A method to assess the toxic or other effects of mixtures of chemicals. (Manuscript)

$$\frac{A_m}{A_i} + \frac{B_m}{B_i} = S, \text{ the sum of biological effects}$$

$$\text{Additive index} = \frac{1}{S} - 1.0 \text{ for } S \leq 1.0 \text{ and}$$

$$\text{Additive index} = [S(-1)] + 1.0 \text{ for } S \geq 1.0$$

where A and B represent concentrations of chemicals. Individual concentrations are designated by i and mixtures of A and B are designated by m.

RESULTS

Effects of QdSO₄:MS-222 Combinations on Test Solutions

QdSO₄:MS-222 solutions are acidic and influence the pH of bioassay water, especially softer waters. A stock solution containing 30 g of QdSO₄ and 60 g of MS-222 in a liter of deionized water has a pH of 1.25. Each chemical decreases the pH of bioassay waters significantly (Marking and Dawson, 1973; Allen and Harman, 1970). The extent of the reduction of pH in waters of various hardnesses by QdSO₄:MS-222 solutions in the ratio of 1:2 is given in table 1. Very soft water is poorly buffered, and the pH is lowered more than 40 percent by a QdSO₄:MS-222 concentration of 35:70 mg/l. In harder waters the pH is

more stable, and in very hard water the pH drops only 12.6 percent at this concentration of the drugs.

The extent to which the pH is decreased appears to be independent of the ratio of the anesthetics. The percentage reduction of the pH was very nearly the same at ratios of 1:2, 1:4, and 1:6 where the total concentration of the two anesthetics was 75 mg/l (table 2).

Species and Sizes of Fish

The toxicity of the mixture of QdSO₄ and MS-222 to nine species of fish is presented in table 3. We selected one ratio of the combination (1:4) to scrutinize the effects of the drugs on various species tested. Lake trout are the most sensitive at all exposures to the 1:4 ratio of QdSO₄ and MS-222 (LC50 = 7.25:29.0 mg/l at 1 hour and 4.23:16.9 mg/l at 96 hours), and coho salmon are the most resistant (LC50 = 11.3:45.0 mg/l at 1 hour and 6.53:26.1 mg/l at 96 hours) (table 3).

Among the warmwater species tested, largemouth bass are the most sensitive to the QdSO₄:MS-222 combination with 1- and 96-hour LC50's of 7.75:31.0 and 5.38:21.5 mg/l, respectively. At a 1-hour exposure to the anesthetics, channel catfish are the most resistant (LC50 = 14.0:56.0 mg/l), but at 96 hours of exposure carp are the most resistant (LC50 = 8.63:34.5 mg/l).

Table 1.--Influence of combinations of QdSO₄:MS-222 (1:2) on the pH of test solutions of various hardnesses

Water hardness	Initial pH	Reduction of pH at QdSO ₄ :MS-222 mixtures (mg/l) of					
		15:30		25:50		35:70	
		Final pH	Percentage reduction	Final pH	Percentage reduction	Final pH	Percentage reduction
very soft.....	6.48	4.53	30.1	3.95	39.0	3.74	42.3
soft.....	7.36	6.57	10.7	5.98	18.8	5.60	23.9
hard.....	7.86	7.19	8.52	6.95	11.6	6.75	14.1
very hard.....	8.20	7.57	7.68	7.34	10.5	7.17	12.6

Table 2.--Influence of three ratios of QdSO₄:MS-222 (75.0 mg/l total) on the pH of test solutions of various hardnesses

Water hardness	Initial pH	Reduction of pH at QdSO ₄ :MS-222 ratios (mg/l) of					
		25:50		15:60		10.7:64.3	
		Final pH	Percentage reduction	Final pH	Percentage reduction	Final pH	Percentage reduction
very soft	6.48	3.95	39.0	3.94	39.2	3.96	38.9
soft	7.36	5.98	18.8	6.03	18.1	6.02	18.2
hard	7.86	6.95	11.6	6.94	11.7	6.96	11.5
very hard	8.20	7.34	10.5	7.35	10.4	7.38	10.0

Larger sizes of coho salmon and brook trout were exposed to the 1:4 ratio of QdSO₄ and MS-222. The 13.2-g coho salmon and 11.6-g brook trout were more resistant than smaller fish of the same species (table 3).

Effects of Temperature and Water Hardness

The toxicity of the drugs to fingerling rainbow trout in soft reconstituted water was not significantly different ($P = 0.05$) at temperatures of 7°, 12°, and 17° C (table 4). The lack of influence by temperature was evident at all exposure periods and concentration ratios tested.

Water hardness apparently influences the toxicity of mixtures of the two anesthetics. The 96-hour LC50's for rainbow trout in very soft water (12 mg/l of total hardness) were significantly ($P = 0.05$) greater than in harder waters (table 4). The decreased activity of the combination in very soft water can possibly be attributed to a decrease in pH as indicated in table 2. At a total of 75 mg/l of QdSO₄ and MS-222 in ratios of 1:2, 1:4, or 1:6, the pH of the test solution dropped to about 4.0. This is below the pKa value of 5.42 for quinaldine (Knight et al., 1955; Sober, 1968) and is very near the pKa value of 3.5 for MS-222 (Maren et al., 1968). The equilibrium for both chemicals, therefore, is shifted in favor of the ionized form which is relatively unavailable to the fish (Sills and Allen, 1971).

In soft water (44 mg/l of total hardness), the combination is more toxic than in very soft water (12 mg/l of total hardness), and the 96-hour LC50's for the 1:4 ratio against rainbow trout are 5.50:22.0 mg/l and 7.63:30.5 mg/l, respectively. At 96 hours the toxicity of the drugs is insignificantly ($P = 0.05$) different in soft, hard (170 mg/l of total hardness) and very hard (300 mg/l of total hardness) water (table 4).

Effects of pH

Although solutions were chemically buffered to specific pH's, the acidic nature of the stock solution of the anesthetics caused a reduction in the pH of the bioassay water. Various amounts of 1 N NaOH were added to each test vessel, depending upon the concentration of the anesthetics, to readjust the pH to the original value. A linear regression of the ml of 1 N NaOH required to readjust the pH to its original value versus the total concentration of the combination of anesthetics produced a slope, intercept, and correlation coefficient of 0.08, 0.0, and 0.9995, respectively. The slope indicates that regardless of the initial pH of the solution, 0.08 ml of 1 N NaOH is required for each mg/l of the combination in order to readjust the pH to its original value.

Tests of the combination of QdSO₄:MS-222 in the ratio of 1:2 at pH 6.5, 7.5, 8.5, and 9.5 indicate the activity is greater at the higher

Table 3.--Toxicity of combinations of QdSO₄ and MS-222 to fish in soft reconstituted water at 12° C

Species	Average weight (g)	Average length (cm)	Ratio of QdSO ₄ :MS-222	LC50 of QdSO ₄ :MS-222 combinations (mg/l) at				
				1 hour	3 hours	6 hours	24 hours	96 hours
Coho salmon....	0.7	4.1	1:6	7.67:46.0	6.98:41.9	6.85:41.1	5.21:31.3	4.93:29.6
Do.....	0.7	4.1	1:4	11.3:45.0	11.0:43.9	10.6:42.4	7.70:30.8	6.53:26.1
Do.....	0.7	4.1	1:2	16.6:33.1	16.6:33.1	16.6:33.1	12.8:25.5	10.3:20.5
Do.....	13.2	10.7	1:4	11.2:44.8	11.2:44.8	11.1:44.2	9.58:38.3	8.55:34.2
Rainbow trout..	0.3	3.1	1:4	10.5:42.0	9.40:37.6	8.05:32.2	6.35:25.4	5.50:22.0
Do.....	0.6	3.8	1:2	16.0:31.9	14.5:29.0	13.5:27.0	9.05:18.1	9.05:18.1
Brown trout....	0.6	3.8	1:6	6.83:41.0	6.50:39.0	6.28:37.7	4.45:26.7	4.45:26.7
Do.....	0.6	3.8	1:4	10.5:42.0	9.05:36.2	8.73:34.9	6.68:26.7	5.73:22.9
Do.....	0.6	3.8	1:2	16.5:33.0	14.9:29.7	13.7:27.3	10.0:20.0	9.15:18.3
Brook trout....	1.2	4.8	1:6	8.58:51.5	8.42:50.5	7.85:47.1	5.95:35.7	4.70:28.2
Do.....	1.2	4.8	1:4	11.0:44.0	10.7:42.7	10.3:41.2	7.23:28.9	6.15:24.6
Do.....	1.2	4.8	1:2	18.9:37.8	18.7:37.3	16.8:33.6	10.9:21.8	9.85:19.7
Do.....	11.6	10.2	1:4	13.2:52.7	11.7:46.6	10.3:41.0	8.70:34.8	7.63:30.5
Lake trout.....	0.5	4.1	1:4	7.25:29.0	6.53:26.1	5.95:23.8	4.25:17.0	4.23:16.9
Carp.....	1.3	4.3	1:6	8.83:53.0	7.22:43.3	6.90:41.4	6.90:41.4	6.68:40.1
Do.....	1.3	4.3	1:4	11.0:44.1	10.2:40.9	9.78:39.1	8.78:35.1	8.63:34.5
Do.....	1.3	4.3	1:2	22.3:44.5	18.1:36.1	16.9:33.8	16.0:32.0	14.7:29.3
Channel catfish	1.8	6.1	1:6	9.25:55.5	9.25:55.5	7.67:46.0	7.23:43.4	5.48:32.9
Do.....	1.8	6.1	1:4	14.0:56.0	11.9:47.7	11.6:46.2	9.25:37.0	7.70:30.8
Do.....	1.8	6.1	1:2	23.4:46.7	20.4:40.7	20.0:40.0	17.2:34.3	11.7:23.3
Bluegill.....	1.5	4.3	1:6	8.50:51.0	5.53:33.2	4.87:29.2	4.87:29.2	4.80:28.8
Do.....	1.5	4.3	1:4	9.25:37.0	7.08:28.3	7.08:28.3	6.93:27.7	6.93:27.7
Do.....	1.5	4.3	1:2	16.1:32.1	13.8:27.5	12.3:24.6	11.0:22.0	11.0:22.0
Largemouth bass	2.8	5.8	1:4	7.75:31.0	6.75:27.0	6.25:25.0	5.50:22.0	5.38:21.5

pH's, especially in longer exposures (table 5). However, the influence of pH in this range is relatively small. The 96-hour LC50's for the combination at pH 6.5 and 9.5 are 8.35:16.7 and 5.90:11.8 mg/l, respectively.

Safety Indices

Safety indices (LC50/EC50) and maximum safety indices (LC1/EC99) were determined for mixtures of QdSO₄:MS-222 (1:2) against rainbow trout at 5-, 10-, 15-, and 30-minute exposures (table 6). The safety indices ranged from 4.23 to 2.60 at 5 and 30 minutes, respectively. The safety index values averaged 1.6 times the corresponding maximum safety indices. As the exposure time increased, the safety margin decreased. Therefore, increased safety is achieved by using concentrations that are effective at shorter exposures.

Quantification of Additive Toxicity

Schoettger and Steucke (1970) indicated that the combination of MS-222 and quinaldine was synergic for anesthetizing fish. The extent of the synergism, or the effect of changing the ratio of the two materials, was not fully defined. The additive index was determined for the data to quantitate the extent of synergism at selected ratios of the two chemicals. If the index is greater than zero, synergism or greater than additive effect is indicated.

Table 7 presents the index values for selected species, exposure periods, and ratios of the two anesthetics. In all cases the index values are greater than zero, and the average of all values is 0.26 indicating that the effect of the two chemicals is greater than additive. Statistical analysis failed to show any significant difference in the additive toxicity between any of the ratios tested ($P = 0.05$).

Table 4.--Toxicity of combinations of QdSO₄ and MS-222 to fingerling rainbow trout at various temperatures and water hardnesses

Temp. (°C)	Water hardness	Ratio of QdSO ₄ :MS-222	LC50 of QdSO ₄ :MS-222 combinations (mg/l) at						
			0.25 hour	0.5 hour	1 hour	3 hours	6 hours	24 hours	96 hours
7.....	soft	1:2	25.3:50.6	16.5:32.9	15.6:31.2	13.8:27.6	11.0:21.9	9.25:18.5	8.40:16.8
Do.....	soft	1:4	--	--	9.80:39.2	9.10:36.4	8.05:32.2	6.35:25.4	5.50:22.0
12.....	soft	1:2	27.5:55.0	16.2:32.3	16.0:31.9	14.5:29.0	13.5:27.0	9.05:18.1	9.05:18.1
Do.....	soft	1:4	--	--	10.5:42.0	9.40:37.6	8.05:32.2	6.35:25.4	5.50:22.0
17.....	soft	1:2	26.5:52.9	15.4:30.8	14.6:29.2	13.5:27.0	12.5:25.0	8.50:17.0	8.35:16.7
Do.....	soft	1:4	--	--	10.4:41.5	8.80:35.2	7.50:29.0	5.73:22.9	5.40:21.6
12.....	very soft	1:2	--	--	--	34.1:68.2	28.0:56.0	16.8:33.6	--
Do.....	very soft	1:4	--	--	14.8:59.0	12.8:51.3	--	9.75:39.0	7.63:30.5
Do.....	hard	1:2	--	15.6:31.1	14.9:29.8	14.1:28.1	12.1:24.1	8.75:17.5	8.75:17.5
Do.....	hard	1:4	--	--	9.00:36.0	8.38:33.5	--	6.00:24.0	5.73:22.9
Do.....	very hard	1:2	--	15.0:30.0	14.1:28.2	14.1:28.2	12.1:24.1	9.90:19.8	9.05:18.1
Do.....	very hard	1:4	--	--	8.63:34.5	8.18:32.7	--	5.80:23.2	5.55:22.2

Table 5.--Toxicity of combinations of QdSO₄ and MS-222 (1:2) to fingerling rainbow trout in soft reconstituted water at 12° C buffered to selected pH's

Time (hours)	LC50 of QdSO ₄ :MS-222 combinations (mg/l) at pH			
	6.5	7.5	8.5	9.5
0.25.....	20.0:40.0	18.0:36.0	24.1:48.1	17.2:34.4
0.50.....	15.3:30.5	15.0:30.0	15.0:30.0	15.5:31.0
1.0.....	13.7:27.4	13.3:26.6	12.5:25.0	10.6:21.1
3.0.....	12.5:25.0	11.5:23.0	9.55:19.1	9.05:18.1
6.0.....	--	--	9.55:19.1	9.05:18.1
24.0.....	9.60:19.2	8.10:16.2	6.20:12.4	6.15:12.3
96.0.....	8.35:16.7	8.10:16.2	6.20:12.4	5.90:11.8

Table 6.--Safety indices for mixtures of QdSO₄:MS-222 (1:2) using rainbow trout in soft reconstituted water at 12° C

Exposure (min)	Concentration of anesthetics (mg/l)				Safety indices	
	LC50	EC50	LC1	EC99	LC50/EC50	LC1/EC99
5.....	56.7	13.4	37.5	14.7	4.23	2.55
10.....	42.2	11.5	36.0	13.8	3.67	2.61
15.....	34.5	10.8	25.1	13.1	3.19	1.92
30.....	27.3	10.5	21.8	12.8	2.60	1.70

Table 7.--Toxicity of QdSO₄ and MS-222 (LC50's in mg/l) individually and in combination and their additive indices at selected exposure periods

Species	Exposure (hours)	Individually		In combination		Ratio	Additive index
		QdSO ₄ ¹	MS-222 ²	QdSO ₄	MS-222		
Rainbow trout...	24	37.0	39.0	6.35	25.4	1:4	0.22
Do.....	24	37.0	39.0	9.05	18.1	1:2	0.41
Do.....	96	31.8	38.4	5.50	22.0	1:4	0.34
Do.....	96	31.8	38.4	9.05	18.1	1:2	0.32
Brown trout.....	24	32.7	38.5	4.45	26.7	1:6	0.21
Do.....	24	32.7	38.5	6.68	26.7	1:4	0.11
Do.....	24	32.7	38.5	10.0	20.0	1:2	0.21
Do.....	96	28.3	43.8	4.45	26.7	1:6	0.30
Do.....	96	28.3	43.8	5.73	22.9	1:4	0.38
Do.....	96	28.3	43.8	9.15	18.3	1:2	0.35
Brook trout.....	24	27.2	50.7	5.95	35.7	1:6	0.08
Do.....	24	27.2	50.7	7.23	28.9	1:4	0.20
Do.....	24	27.2	50.7	10.9	21.8	1:2	0.20
Do.....	96	22.2	50.0	4.70	28.2	1:6	0.29
Do.....	96	22.2	50.0	6.15	24.6	1:4	0.30
Do.....	96	22.2	50.0	9.85	19.7	1:2	0.19
Lake trout.....	24	16.3	33.8	4.25	17.0	1:4	0.31
Do.....	96	15.5	32.0	4.23	16.9	1:4	0.25
Bluegill.....	24	36.8	45.7	4.87	29.2	1:6	0.30
Do.....	24	36.8	45.7	6.93	27.7	1:4	0.26
Do.....	24	36.8	45.7	11.0	22.0	1:2	0.28
Do.....	96	32.0	45.7	4.80	28.8	1:6	0.28
Do.....	96	32.0	45.7	6.93	27.7	1:4	0.22
Do.....	96	32.0	45.7	11.0	22.0	1:2	0.21
Largemouth bass.	24	16.0	47.0	5.50	22.0	1:4	0.23

¹ From (Marking and Dawson, 1973)² From (Marking, 1967)

DISCUSSION

The pattern of toxic response of the mixture of QdSO₄:MS-222 among various species and sizes of fish is similar to that of each component when tested individually (Marking, 1967; Marking and Dawson, 1973). In both cases lake trout were the most sensitive of the coldwater species tested and largemouth bass were the most sensitive of the warmwater species tested. Also, larger fish

of the same species were more resistant than smaller ones.

The effect of temperature, however, does not show a similar pattern when tested individually. Temperature had very little effect on the toxicity of the mixture of the anesthetics to rainbow trout. However, when MS-222 was tested individually the trout were more resistant at lower temperatures. This

was true also of the QdSO_4 for longer exposures, but the trend was reversed in 1- to 6-hour exposures.

Tests of the anesthetic mixture at adjusted pH's of 6.5, 7.5, 8.5, and 9.5 indicated the pH had only a slight influence on the toxicity of the anesthetics in this range. This is not surprising considering the pK_a value of each of the components is more than one pH unit below the lowest pH tested. According to the Henderson-Hasselbach equation, even at pH 6.5, 92.3 percent of the QdSO_4 and 99.9 percent of the MS-222 would be un-ionized. The un-ionized forms of both molecules are lipid-soluble, thereby making both anesthetics potentially available to the fish (Sills and Allen, 1971).

On the other hand, there was a significant decrease in the pH of poorly buffered solutions. This is because QdSO_4 is a water-soluble salt of quinaldine, and MS-222 is a water-soluble salt of *m*-aminobenzoic acid ethyl ester. Being water-soluble, the salt forms are easier to handle, but the sulfuric acid from QdSO_4 and the methane sulfonic acid from MS-222 are strong acids. If the anesthetic mixture were used in soft, unbuffered water, the pH may go below 6.5, and there would be a substantial decrease in both toxicity and efficacy.

The toxicity of the anesthetics is increased when they are combined as indicated by an average additive index of 0.26. The increased toxicity of the combination would be hazardous when the desired effect is sedation and not mortality. However, when the additive index formula is applied to information presented by Berger (1969) on the efficacy of the mixture as an anesthetic, a value of 0.29 is obtained. The index for toxicity and the index for anesthesia are both greater than one, thus indicating that although the mixture is more toxic it also is more effective as an anesthetic. The important advantage in using the mixture is in combining the rapid, deep anesthetization of MS-222 and the long, safe exposure time of QdSO_4 .

CONCLUSIONS

1. Ninety-six hour LC_{50} 's for the 1:4 ratio of QdSO_4 :MS-222 among nine species of fish ranged from 4.23:16.9 mg/l for lake trout to 8.63:34.5 mg/l for carp in soft reconstituted water at 12° C.
2. Larger fish are generally more resistant to the combined anesthetics than smaller fish.
3. The toxic effect of the combination is greater than additive as indicated by an average additive index of 0.26. The additive toxicity of QdSO_4 :MS-222 ratios of 1:2, 1:4, and 1:6 were insignificantly different ($P = 0.05$).
4. The toxicity of the drugs to fingerling rainbow trout was not influenced by temperature changes from 7° to 17° C.
5. The combination of anesthetics is slightly less toxic in solutions adjusted to pH 6.5 than in solutions adjusted to pH 9.5. The lower pH probably reduces the concentration of the active, un-ionized form of the molecules.
6. The mixture is less toxic in very soft water than in harder water, but the decreased pH in very soft water is considered responsible for the reduced activity.
7. Safety indices indicate that the safety margin is greater at shorter exposures.

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54. The Efficacy of Quinaldine Sulfate: MS-222 Mixtures for the Anesthetization of Freshwater Fish

By Philip A. Gilderhus, Bernard L. Berger,
Joe B. Sills, and Paul D. Harman



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THE EFFICACY OF QUINALDINE SULFATE: MS-222 MIXTURES FOR THE ANESTHETIZATION OF FRESHWATER FISH

By Philip A. Gilderhus, Bernard L. Berger, Joe B. Sills,
and Paul D. Harman

ABSTRACT.--Combinations of quinaldine sulfate (QdSO_4) and MS-222 were tested for their efficacy in anesthetizing 14 species of freshwater fish. The combinations induced rapid and deep anesthesia as does MS-222 and permitted long safe holding times as does QdSO_4 . The concentrations of the combined anesthetics needed were considerably lower than those needed when MS-222 is used alone. Most salmonids tested required concentrations of 10:20 to 10:40 mg/l (QdSO_4 :MS-222) for effective anesthetization. Warmwater species generally required higher concentrations of 10:40 to 20:75 mg/l. Large adult fish usually required higher concentrations than smaller fish.

Both compounds lower the pH of the solution, and at pH's approaching 6.0 or below the combinations were much less effective. In soft waters where the pH was lowered to that point, buffering the pH back to 6.5 or higher restored the activity of the anesthetics.

INTRODUCTION

The individual attributes and use patterns of quinaldine and MS-222 as anesthetics for fish have been well documented (Schoettger and Julin, 1967, 1969). Schoettger and Steucke (1970) tested mixtures of quinaldine and MS-222 against rainbow trout¹ and northern pike and found the combinations to possess most of the attributes of both anesthetics. Furthermore, substantially less of each component was necessary when they were used in combination. The combination in concentrations from 5:20 mg/l (quinaldine: MS-222) for rainbow trout to 20:60 mg/l for northern pike, rapidly anesthetized the fish and permitted them to be held safely in the chemical solution for at least 60 minutes.

Most recently Allen and Sills (1973) synthesized quinaldine sulfate (QdSO_4), a salt of quinaldine which is more convenient to use than

quinaldine, because it is water soluble. The efficacy of QdSO_4 was found to be essentially the same, on an active ingredient basis, as that of quinaldine (Gilderhus et al. 1973).

Since QdSO_4 is a crystalline material, it appeared to be ideal for use in combination with MS-222. The two compounds could be blended together and stored or marketed as a ready-to-use mixture. Using the data of Schoettger and Steucke (1970) as a starting point, our objectives were to determine the effective concentrations and ratios of concentrations of the combined anesthetics for 14 species of fish, and evaluate the influences of water quality and temperature on the efficacy of the anesthetics.

METHODS AND MATERIALS

The quinaldine sulfate (QdSO_4) used in these tests was synthesized at the Southeastern Fish Control Laboratory, Warm Springs, Ga. The MS-222 (methane sulfonate of meta-aminobenzoic acid ethyl ester) was Finquel^(R), marketed by Ayerst Laboratories, Inc.

¹ The common and scientific names of the fish used in the present study are given in table 1.

The anesthetics for each combination were weighed individually and either introduced directly into the test vessel or mixed into water solution in a flask and then introduced into the test vessel. The same procedures were used for both laboratory and field tests.

Tests were conducted at the Fish Control Laboratories, La Crosse, Wis., and Warm Springs, Ga., depending on the availability of test fish. Fingerling-size fish were exposed to the anesthetics in 15-l glass jars, and 45- and 100-l polyethylene tanks were used for tests with larger fish. The temperature was maintained by placing the test vessel in a circulating water bath equipped with heating or cooling equipment.

The efficacy of the combined anesthetics was evaluated against five species of salmonids and nine species of warmwater fish (table 1). The fish for laboratory tests were obtained from federal or state fish hatcheries except

for small coho salmon and rainbow trout which were hatched and reared at the La Crosse laboratory. All fish used in laboratory tests were maintained as described by Hunn et al. (1968). They were acclimated to the test conditions for 16 to 24 hours before the anesthetics were added. Tests were also conducted against the five species of salmonids, northern pike, and walleyes at fish hatcheries during their spawning and marking operations.

The laboratory tests were conducted in well, city, and reconstituted waters at La Crosse and in limed spring water at Warm Springs (table 2). The efficacy of the anesthetics at selected pH's was assessed in reconstituted waters in which the pH was adjusted with a KH_2PO_4 -NaOH buffer system (Marking, 1969). In some tests where the anesthetic chemicals lowered the pH of the water below the point where they were effective, the pH was raised by adding NaHCO_3 . For example, to raise the pH of 45 l of water to 7.0, 1.9 and 11.0 g of NaHCO_3 were added to waters of pH 5.3 and 3.8, respectively.

Laboratory tests were conducted at 7°, 12°, and 27° C at La Crosse and at 19° C at Warm Springs. Field tests were conducted at the

Table 1.--Species of fish used in tests of the efficacy of QdSO_4 :MS-222 mixtures as anesthetics for fish

Common name	Scientific name
Coho salmon	<u>Oncorhynchus kisutch</u>
Rainbow trout	<u>Salmo gairdneri</u>
Brown trout	<u>Salmo trutta</u>
Brook trout	<u>Salvelinus fontinalis</u>
Lake trout	<u>Salvelinus namaycush</u>
Northern pike	<u>Esox lucius</u>
Muskellunge	<u>Esox Masquinongy</u>
Carp	<u>Cyprinus carpio</u>
White amur	<u>Ctenopharyngodon idellus</u>
White sucker	<u>Catostomus commersoni</u>
Black bullhead	<u>Ictalurus melas</u>
Channel catfish ...	<u>Ictalurus punctatus</u>
Bluegill	<u>Lepomis macrochirus</u>
Largemouth bass ...	<u>Micropterus salmoides</u>
Walleye	<u>Stizostedion vitreum</u>

Table 2.--Characteristics of waters used for laboratory tests of QdSO_4 :MS-222 mixtures as anesthetics for fish

Water type	pH	Total	
		Alkalinity (mg/l)	Hardness (mg/l)
well	7.5-8.0	232-262	238-371
city	7.4-8.2	209-250	289-340
spring ¹	6.8-7.0	(²)	20
<u>Reconstituted</u>			
very soft	6.4-6.8	10-13	10-13
soft.....	7.2-7.6	30-35	40-48
very hard.....	8.0-8.4	225-245	280-320

¹ CaO added to water to prevent osmotic shock in the fish.

² Not analyzed.

existing water temperatures of the hatchery water supplies (table 3).

Schoettger and Julin (1967) defined loss of equilibrium, stage 2, as the degree of anesthesia at which locomotion ceases, and opercular rate slows, but there is still some reflex response to pressure on the caudal peduncle. We found that fish anesthetized by mixtures of QdSO₄:MS-222 were easily handled when in loss of equilibrium, stage 2. Therefore, the tests were designed to determine the concentrations and

ratios of QdSO₄:MS-222 which would anesthetize the fish to loss of equilibrium, stage 2, in approximately 4 min or less.

RESULTS

Behavior of the Fish

Fish exposed to the combination of anesthetics generally go through a period of 20 to 30 seconds of normal swimming before becoming sedated. The progression of anesthesia is rapid from sedation to loss of equilibrium, stage 2, at which point it

Table 3.--Characteristics of hatchery water supplies used in tests of QdSO₄:MS-222 mixtures as anesthetics for fish

Location	Species tested	Water temp. (°C)	pH	Total	
				Alkalinity (mg/l)	Hardness (mg/l)
Platte River SFH ¹ Michigan	Coho salmon	6	7.8	150	168
Manchester NFH ² Iowa	Rainbow trout	9	7.5	172	215
Manchester NFH Iowa	Brown trout	8	(³)	--	--
Osceola SFH Wisconsin	Brook trout	9	8.1	171	208
Crystal Springs SFH Minnesota	Lake trout (adult)	8	7.5	257	280
Jordan River NFH Michigan	Lake trout (fingerling)	7	7.6	120	120
Lansing SFH Iowa	Northern pike	5	9.2	141	144
Valley City NFH North Dakota	Muskellunge	18	7.9	179	213
Lansing SFH Iowa	Walleye	10	9.2	133	164

¹ State Fish Hatchery.

² National Fish Hatchery.

³ Same water supply as used for rainbow trout. Analysis not done.

either slows or stops. As with quinaldine sulfate alone (Gilderhus et al. 1973), the combination of chemicals rarely induces total loss of reflex. However, the fish are easily handled while in loss of equilibrium, stage 2, and the reflexes which are retained are usually weak and of little consequence to the handler.

Efficacy of the Anesthetics

Combinations of QdSO_4 :MS-222 proved to be effective anesthetics for all species of fish on which they were tested. Concentrations of 10:20 mg/l (QdSO_4 :MS-222) were the lowest which were effective for four species of trout in laboratory tests. Coho salmon were slightly more sensitive, requiring a combination of 5:20 mg/l. The salmonids recovered rapidly in fresh water, and they recovered faster in warmer water, requiring up to 4.7 min at 17° C and 20 min at 7° C (table 4).

The larger salmonids exposed to the anesthetics at field stations were somewhat more resistant than the smaller fish exposed in the laboratory, requiring concentrations of 10:20 to 10:40 mg/l (table 5). Brook and lake trout

required the highest concentrations under field conditions to subdue them to a handleable condition for artificial spawning. This agrees with Schoettger and Julin (1967) who found brook and lake trout to be more resistant than other trouts to MS-222 alone.

The combination of drugs was less active on most of the species of warmwater fish than on salmonids, and all but small walleyes required higher concentrations. Black bullheads were the most resistant in laboratory tests requiring concentrations of 20:75 mg/l (table 6). Northern pike, carp, and white suckers were the next most resistant requiring 20:50 mg/l to 20:75 mg/l. Small walleyes, the most sensitive fish, were anesthetized by a combination of 5:15 mg/l (QdSO_4 :MS-222).

Higher concentrations of the combined anesthetics also were required for larger specimens of the warmwater species. Large northern pike required 20:75 mg/l and small northern pike 20:50 mg/l; large channel catfish required 40:60 mg/l and small channel catfish 20:50 mg/l; large walleyes required 10:30 mg/l and small walleyes 5:15 mg/l.

Table 4.--Efficacy of QdSO_4 :MS-222 combinations as anesthetics for salmonids in laboratory tests

Species	Mean weight (g)	No. of fish	Temp. (°C)	Water type	Concentration (mg/l)	Loss of equilibrium stage 2 (min)	Length of exposure (min)	Recovery in fresh water (min)
Coho salmon...	19	15	7	well	5:20	1.5-2.6	15-60	3.2-5.5
Do.....	16	15	12	soft	10:20	1.4-3.0	15-60	3.1-5.3
Do.....	19	10	12	well	5:20	1.3-1.8	15-30	2.0-3.0
Rainbow trout.	25	15	7	well	10:20	0.9-1.5	15-60	3.0-7.8
Do.....	1.4	15	12	soft	10:40	1.3-4.5	15-60	2.7-10.0
Do.....	1.5	10	12	well	10:20	0.5-0.6	5.5-15	2.0-3.2
Do.....	18	10	12	very hard	10:20	0.9-1.5	15-30	2.0-3.8
Do.....	0.9	15	17	soft	10:20	1.4-3.3	15-60	1.5-3.0
Do.....	0.9	15	17	¹ pH-7.6 soft	10:20	1.8-2.9	15-60	2.0-4.5
Do.....	25	15	17	¹ pH-8.5 well	10:20	0.8-0.9	15-60	1.0-4.0
Brown trout...	18	10	7	well	10:20	1.1-1.5	15-30	3.0-6.3
Do.....	28	15	12	well	10:20	0.9-1.5	15-60	3.0-7.0
Do.....	16	15	17	well	10:20	1.0-1.3	15-60	2.0-3.8
Brook trout...	27	15	7	well	10:20	0.9-1.4	15-60	3.5-9.4
Do.....	1.1	15	12	soft	10:20	1.0-3.5	5.5-15	1.5-2.6
Do.....	27	15	12	well	10:20	0.9-1.4	15-60	3.0-5.0
Lake trout....	25	10	7	well	10:20	1.6-2.6	15-30	7.0-20.0
Do.....	30	15	12	well	10:20	0.9-1.7	15-60	3.0-11.0
Do.....	25	15	17	well	10:20	1.0-2.5	15-60	2.5-4.7

¹ pH adjusted with buffers.

Table 5.--Efficacy of QdSO₄:MS-222 combinations as anesthetics for fish at field stations

Species and location	Mean weight (kg)	No. of fish	Effective concentration (mg/l)	Time (min) to	
				Loss of equilibrium Stage 2	Recovery in fresh water
Coho salmon Platte River SFH ¹	3.5	15	5:10	0.8-2.3	3.0-4.0
Rainbow trout Manchester NFH ²	3.2	74	10:30	1.2-3.0	3.2-5.0
Brown trout Manchester NFH	2.6	62	5:30	2.0-3.2	1.0-4.5
Brook trout Osceola SFH	1.0	57	10:40	1.2-1.5	2.2-5.5
Lake trout Crystal Springs SFH	2.5	55	10:40	3.0-4.0	4.0-5.0
Lake trout Jordan River NFH	0.01	2,700	10:20	1.2-2.0	4.0-5.5
Northern pike Lansing SFH	1.1	12	20:50	2.5-8.0	8.8-22.0
Muskellunge Valley City NFH	2.3	8	20:50	1.2-1.5	3.0-6.6
Walleye Lansing SFH	0.9	10	10:30	2.5-3.5	6.0-10.0

¹ State Fish Hatchery.² National Fish Hatchery.Table 6.--Efficacy of QdSO₄:MS-222 combinations as anesthetics for warmwater fish in laboratory tests

Species	Mean weight (g)	No. of fish	Temp. (°C)	Water type	Concentration (mg/l)	Loss of equilibrium Stage 2 (min)	Length of exposure (min)	Recovery in fresh water (min)
Northern pike.....	10	15	12	well	10:40	2.4-3.1	15-60	5.5-11.0
Do.....	1791	18	12	well	20:75	3.0-5.0	30	19.0-26.0
Do.....	115	10	17	very soft	20:50	1.7-2.5	5.5-15	3.5-5.9
Do.....	10	15	17	well	10:40	1.8-2.0	15-60	4.5-5.0
Do.....	115	15	22	very soft	20:50	0.9-2.0	5.5-30	3.2-8.5
Carp.....	387	20	12	well	20:50	2.1-3.3	5.5-15	4.5-9.0
Do.....	387	10	27	well	20:50	1.6-2.3	5.5-15	2.5-6.5
White amur.....	227	3	19	spring	20:40	1.5-1.8	30	4.0-5.0
White sucker.....	138	10	12	well	20:50	1.7-2.0	5.5	5.1-7.0
Black bullhead.....	208	10	12	well	20:75	3.3-4.1	5.5-15	10.0-22.0
Do.....	208	10	17	well	20:75	3.5-4.1	15	4.5-16.0
Do.....	129	10	27	well	20:50	2.5-3.1	5.5-15	4.2-6.5
Channel catfish.....	1.8	10	12	well	30:30	1.5-2.3	5.5-15	2.2-14.0
Do.....	1.5	10	17	well	20:50	0.6-1.5	15-30	¹ 1.8-3.5
Do.....	1.8	10	17	very hard	20:50	1.5-2.5	5.5-15	2.0-2.8
Do.....	1316	30	19	spring	40:60	2.0-3.0	30	3.5-7.5
Bluegill.....	77	14	17	well	10:40	1.4-2.1	15-60	5.0-8.2
Do.....	135	49	19	spring	10:40	2.5-3.5	30	1.5-3.0
Do.....	80	15	27	well	10:40	1.1-1.2	5.5-15	1.7-3.0
Largemouth bass.....	12	5	17	well	20:50	1.0-1.1	15	5.0-6.5
Do.....	15	10	17	very hard	20:50	0.8-1.5	5.5-15	2.7-10.0
Do.....	1044	30	19	spring	20:40	1.5-3.1	30	2.5-9.5
Walleye.....	1.1	15	12	well	5:15	1.5-3.3	15-60	5.0-26.0

¹ Some fish killed by 30-minute exposure.

The time required for the fish to recover in fresh water was inversely related to the water temperature with longer times being necessary in colder water. Most fish recovered in less than 10 min, but some needed up to 26 min (table 6).

Water Quality

The efficacy of the combined anesthetics was affected by the chemical characteristics of the water. Both of the compounds are acidic, lowering the pH of the water to which they are added (Allen and Harman, 1970; Marking and Dawson, 1973). We found that in anesthetic solutions of about pH 6 or below, the anesthetics were diminished in efficacy, depending on the concentration. The lowering of the pH was critical only when the anesthetics were placed in soft or very soft, unbuffered water. With rainbow trout, 10:20 mg/l were effective in well water and not effective in very soft water at 12° C. The combination anesthetized northern pike at 10:30 mg/l in well water; whereas 20:75 mg/l were ineffective in soft water at 12° C. Increasing the concentration sometimes compensated for the loss of activity in soft waters, but adding NaHCO₃ until the pH of the solution was 6.5 or higher, assured satisfactory activity.

Water Temperature

The water temperature did not decisively or consistently affect the efficacy of the combined anesthetics. The concentrations needed for effective anesthetization of salmonids were the same over a wide range of water temperatures. There was some indication that northern pike and black bullheads might be anesthetized by lower concentrations at higher temperatures but the results were not conclusive. Inconsistent results related to temperature were not surprising. The efficacy of MS-222 apparently is affected by temperature (Schoettger and Julin, 1967), whereas the efficacy of quinaldine is not (Schoettger and Julin, 1969). The recovery time for fish was more consistently related to temperature with recovery being more rapid at higher temperatures.

Repeated Exposure

Repeated anesthetization of the same fish does not appear to affect the sensitivity of the

fish to the combined anesthetics. A group of ten 20-cm rainbow trout was anesthetized 11 times in 15 days by a 20:50 mg/l solution and anesthetization and recovery times were unaffected.

Repeated Use of Solutions

The repeated use of solutions of QdSO₄:MS-222 was evaluated during the fin clipping of lake trout at Jordan River NFH. We found that 1,800 fish (a total of 25 kg) could be anesthetized in 8 l of a 10:20-mg/l solution before the solution had to be spiked or replaced.

Apparently, raising the concentration slightly from that normally used will help ensure continued effectiveness for a period of several days. Fifty l of a 20:100-mg/l solution were used for 3 days to anesthetize 2.2-3.6 kg northern pike. A total of 135 pike was anesthetized the first day, 125 the second, and 120 the third without noticeable loss of activity of the solution.

DISCUSSION

The combinations of QdSO₄ and MS-222 combined the attributes of the individual anesthetics and induced anesthesia more effectively than QdSO₄ alone and more safely than MS-222 alone. Fish can be safely held for 1 hour or more in concentrations which effectively anesthetize the respective species. This is in contrast to the 5.5- to 12-minute safe holding times for salmonids in MS-222 given by Schoettger and Julin (1967). An exception was channel catfish which suffered mortalities after 30 min of exposure to 20:50 mg/l, the lowest effective concentration. The long safe holding time afforded by the combination is a distinct advantage because more fish can be anesthetized at one time without danger to the last ones handled. Whereas the combinations do not consistently induce total loss of reflex as does MS-222, they do make fish more handleable than does QdSO₄ alone.

The concentrations of the drugs used in combination represent a substantial saving of chemicals over the concentrations necessary when they are used alone. For example, when used alone for salmonids, the concentrations

needed are 80 to 100 mg/l of MS-222 or 25 mg/l of QdSO₄. When used in combination, the concentrations necessary to anesthetize salmonids are 10:20 mg/l (QdSO₄:MS-222).

The water chemistry appears to be the only factor which consistently influences the efficacy of the combined anesthetics. Apparently, the two chemicals are affected differently, but both have reduced activity in very soft water. Both compounds lower the pH of the water, contributing to the ionization and inactivation of QdSO₄ at pH's below 6. MS-222 is less effective in soft waters, apparently because the lack of calcium ions induces osmotic stress in the fish which interferes with the activity of the anesthetic (Schoettger and Julin, 1967).

CONCLUSIONS

1. Combinations of QdSO₄ and MS-222 effectively anesthetize a wide variety of fishes.
2. The combinations possess the attributes of both anesthetics--that is, the long safe holding time with QdSO₄ and the rapid anesthetization with MS-222.
3. Combining the anesthetics greatly reduces the concentrations over those necessary when they are used alone.
4. Higher concentrations of the combination are generally needed for large adult fish than for small, immature fish.
5. The combination is relatively ineffective if it lowers the pH of the water to 6 or below. This is more prone to occur in soft or unbuffered water.
6. If the combined anesthetics lower the pH of the solution to near 6 or below, the pH should be raised to 6.5 or higher with NaHCO₃ or another satisfactory buffer.

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**55. Residues of Quinaldine and MS-222 in Fish Following
Anesthesia with Mixtures of Quinaldine
Sulfate:MS-222**

By Joe B. Sills, John L. Allen, Paul D. Harman,
and Charles W. Luhning



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Bureau of Sport Fisheries and Wildlife**

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RESIDUES OF QUINALDINE AND MS-222 IN FISH FOLLOWING ANESTHESIA WITH MIXTURES OF QUINALDINE SULFATE:MS-222

By Joe B. Sills, John L. Allen, Paul D. Harman,
and Charles W. Luhning

ABSTRACT.--Residues of quinaldine and MS-222 in 10 species of fish exposed to mixtures of quinaldine sulfate and MS-222 were determined using gas chromatography and spectrophotometry for quinaldine and colorimetry for MS-222. The residue concentrations of quinaldine and MS-222 decreased rapidly following withdrawal from the anesthetics. The mean concentration of 0-hour residues of quinaldine ranged from 0.15 to 6.92 $\mu\text{g/g}$ depending on concentration, temperature, length of exposure, and species. The mean concentration of 0-hour residues of MS-222 ranged from 1.9 to 27.3 $\mu\text{g/g}$ and decreased to near the background reading of the controls after 24 hours of withdrawal. The fish exposed to the same concentration of the mixture of anesthetics for 15 minutes generally contained higher concentrations of residues than those exposed for 5.5 minutes. Two weight groups of brook trout were exposed to the same concentrations of the mixed anesthetics for the same length of time. The smaller fish contained 1.22 times higher concentration of quinaldine residue and 1.43 times higher concentration of MS-222 residue than the larger fish.

INTRODUCTION

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) and quinaldine (2-methylquinoline) are effective and widely-used fish anesthetics (Schoettger and Julin, 1967, 1969). The choice of which anesthetic to use depends upon the specific properties that are desired. MS-222 causes rapid immobility, but fish are unable to tolerate long exposures. On the other hand, quinaldine is tolerated for long periods, but does not completely block reflex movement. Schoettger and Steucke (1970) found that mixtures of these drugs offer advantages over the use of each separately. The mixture produced rapid immobility and prolonged toleration at slightly reduced concentrations of each drug.

Allen and Sills (1973) prepared a water-soluble form of quinaldine by forming its sulfate salt. The toxicity of quinaldine sulfate (QdSO_4) to fish was determined by Marking and Dawson (1973), and its efficacy as a fish

anesthetic was determined by Gilderhus et al. (1973a). The toxicity of the anesthetic mixture (quinaldine sulfate:MS-222) was determined by Dawson and Marking (1973). Gilderhus et al. (1973b) determined the efficacy of the combination anesthetic in the laboratory and under field conditions.

Residue data on four salmonids¹ and channel catfish anesthetized with MS-222 have been reported (Walker and Schoettger, 1967; Schoettger et al., 1967). Allen et al. (1972) determined MS-222 residues in northern pike, muskellunge, and walleye anesthetized with MS-222. Sills et al. (1973) determined residues of quinaldine in five species each of coldwater and warmwater fish following anesthesia with quinaldine sulfate. Sills and Harman (1970) determined quinaldine residues in striped bass (Morone saxatilis) following anesthesia with quinaldine sulfate.

¹The common and scientific names of fish used in the present study are given in table 1.

Before the mixture of quinaldine sulfate and MS-222 can be registered for general use, more information is needed about the fate of quinaldine and MS-222 in fish tissues. Therefore, this study was undertaken to measure the concentration and persistence of quinaldine and MS-222 residues in five species each of coldwater and warmwater fish following anesthesia with efficacious concentrations of the mixture.

METHODS AND MATERIALS

Ten species of fish (table 1) were exposed to quinaldine sulfate and MS-222 (Gilderhus et al., 1973b). Temperatures of treatment ranged from 7° to 19° C, and exposure times ranged from 5.5 to 30 minutes. A wide range of concentrations was necessary, because of the variety of species and temperatures involved.

Withdrawal times began when exposed fish were placed in fresh, flowing water for recovery. At least three fish were collected for residue analysis at 0, 1, 2, 4, either 6 or 8, and 24 hours. Samples of muscle tissue were collected and held frozen until analyzed. Whole fillets were homogenized after thawing to obtain representative samples of edible tissue.

Table 1.--Species of fish analyzed for quinaldine and MS-222 residues following anesthesia with mixtures of quinaldine sulfate and MS-222

Common Name	Scientific Name
Coho salmon	<u>Oncorhynchus kisutch</u>
Brown trout	<u>Salmo trutta</u>
Rainbow trout	<u>Salmo gairdneri</u>
Lake trout	<u>Salvelinus namaycush</u>
Brook trout	<u>Salvelinus fontinalis</u>
Northern pike	<u>Esox lucius</u>
Channel catfish ...	<u>Ictalurus punctatus</u>
Largemouth bass ...	<u>Micropterus salmoides</u>
Bluegill	<u>Lepomis macrochirus</u>
Walleye	<u>Stizostidion vitreum</u>

The samples were analyzed by the colorimetric method of Walker and Schoettger (1967) for MS-222 residue and by the gas chromatographic and U.V. spectrophotometric methods of Allen and Sills (1970a and 1970b) for quinaldine residue. The minimum detectable concentration of the quinaldine methods is 0.01 µg/g and the minimum detectable concentration of the MS-222 method is 0.1 µg/g. Residues of quinaldine less than 0.01 µg/g are reported as zero. The minimum detectable concentration of the MS-222 method is limited also by the background aromatic amines, and all MS-222 results include these.

RESULTS

Coho salmon

Spawning-migrant coho salmon from Lake Michigan were exposed to a mixture of 5 mg of quinaldine sulfate and 10 mg of MS-222 per liter of water at 12° C for 5.5 and 15 minutes (table 2). MS-222 residues ranged from mean concentrations of 1.9 to 3.3 µg/g at the 0-hour interval and decreased to a background level of the controls or slightly above at the 24-hour withdrawal interval. Quinaldine residues ranged from mean concentrations of 0.15 to 0.51 µg/g at the 0-hour interval to zero after 4 to 8 hours of withdrawal. The coho salmon were the largest fish tested.

Brown trout

Brown trout (table 2) were exposed to a mixture of 5 mg of quinaldine sulfate and 30 mg of MS-222 per liter of water at 12° C for 5.5 and 15 minutes. MS-222 residues ranged from mean concentrations of 7.2 to 14.6 µg/g at the 0-hour withdrawal and were within background levels after 8 to 24 hours. Quinaldine residues ranged from mean concentrations of 0.33 to 0.63 µg/g at the 0-hour withdrawal, and were down to zero after 8 hours of withdrawal.

Rainbow trout

Hatchery-reared rainbow trout were tested the most extensively (table 3). Those exposed at 7° C to a mixture of 5 mg of quinaldine sulfate and 30 mg of MS-222 per liter of water for 15 minutes contained a mean concentration

Table 2.--Residues of anesthetics in muscle tissue of coho salmon and brown trout treated with mixtures of quinaldine sulfate (QdSO₄) and MS-222 at 12° C for 5.5 and 15 minutes

	Treatment			Mean weight (kg)	Residues (μ/g) ¹			
	QdSO ₄ (mg/l)	MS-222 (mg/l)	Exposure (min)		Quinaldine		MS-222	
					Mean	Range	Mean	Range
Coho salmon								
Control.....	0	0	0	2.03	0.00	0.00-0.00	1.1	0.6-1.4
0-hour.....	5	10	5.5	3.82	0.15	0.09-0.18	3.3	1.6-4.2
1-hour.....	5	10	5.5	1.53	0.10	0.07-0.14	1.5	1.4-1.6
2-hour.....	5	10	5.5	3.20	0.02	0.01-0.03	1.5	1.4-1.6
4-hour.....	5	10	5.5	3.91	0.00	0.00-0.00	0.9	0.6-1.4
8-hour.....	5	10	5.5	2.71	0.00	0.00-0.00	1.5	1.4-1.6
24-hour.....	5	10	5.5	1.57	0.00	0.00-0.00	1.1	0.6-2.0
Brown trout								
0-hour.....	5	10	15	3.91	0.51	0.34-0.65	1.9	1.6-2.0
24-hour.....	5	10	15	4.37	0.00	0.00-0.00	1.8	1.0-2.6
Brown trout								
Control.....	0	0	0	0.58	0.00	0.00-0.00	0.9	0.6-1.4
0-hour.....	5	30	5.5	0.44	0.33	0.23-0.40	7.2	5.0-9.0
2-hour.....	5	30	5.5	0.56	0.01	0.01-0.02	1.0	1.0-1.0
8-hour.....	5	30	5.5	0.50	0.00	0.00-0.00	0.6	0.6-0.6
0-hour.....	5	30	15	0.41	0.63	0.55-0.75	14.6	11.4-17.0
2-hour.....	5	30	15	0.48	0.03	0.02-0.04	1.3	1.0-1.4
4-hour.....	5	30	15	0.56	0.01	0.01-0.02	0.7	0.6-1.0
8-hour.....	5	30	15	0.53	0.00	0.00-0.00	1.0	0.6-1.4
24-hour.....	5	30	15	0.51	0.00	0.00-0.00	0.9	0.6-1.0

¹ Each mean value represents the average of three analyses.

Table 3.--Residues of anesthetics in muscle tissue of rainbow trout anesthetized with mixtures of quinaldine sulfate (QdSO₄) and MS-222 at 7°, 12°, and 17° C for 5.5 and 15 minutes

Withdrawal interval	Treatment				Mean weight (g)	Residues (μg/g) ¹			
	QdSO ₄ (mg/l)	MS-222 (mg/l)	Exposure (min)	Temp. (°C)		Quinaldine		MS-222	
						Mean	Range	Mean	Range
Control.....	0	0	0	7	384	0.00	0.00-0.00	0.7	0.5- 1.0
0-hour.....	5	30	15	7	496	0.29	0.25-0.35	5.2	3.4- 7.6
1-hour.....	5	30	15	7	532	0.24	0.20-0.28	2.4	2.0- 2.8
2-hour.....	5	30	15	7	483	0.13	0.07-0.18	1.9	1.4- 2.8
4-hour.....	5	30	15	7	445	0.02	0.01-0.03	0.8	0.6- 1.0
Control.....	0	0	0	12	135	0.00	0.00-0.00	0.5	0.5- 0.5
0-hour.....	10	40	5.5	12	116	0.72	0.64-0.88	11.9	10.0-13.0
1-hour.....	10	40	5.5	12	148	0.22	0.14-0.29	1.9	1.6- 2.0
2-hour.....	10	40	5.5	12	168	0.03	0.02-0.04	0.6	0.6- 0.6
4-hour.....	10	40	5.5	12	172	0.02	0.01-0.02	0.6	0.5- 0.6
8-hour.....	10	40	5.5	12	155	0.02	0.01-0.03	0.5	0.5- 0.6
24-hour.....	10	40	5.5	12	100	0.00	0.00-0.00	1.0	1.0- 1.0
0-hour.....	10	40	15	12	126	1.44	0.80-2.37	17.0	13.0-24.0
8-hour.....	10	40	15	12	152	0.01	0.01-0.01	0.9	0.6- 1.0
24-hour.....	10	40	15	12	147	0.00	0.00-0.00	1.0	1.0- 1.0
Control.....	0	0	0	17	135	0.00	0.00-0.00	0.7	0.6- 1.0
0-hour.....	5	30	5.5	17	420	0.53	0.48-0.58	6.9	5.2- 8.6
1-hour.....	5	30	5.5	17	412	0.23	0.20-0.26	1.0	0.6- 1.4
2-hour.....	5	30	5.5	17	467	0.12	0.08-0.15	0.8	0.6- 1.0
4-hour.....	5	30	5.5	17	390	0.03	0.02-0.03	0.5	0.5- 0.6
0-hour.....	5	30	15	17	126	1.05	0.72-1.90	16.5	11.6-21.4
1-hour.....	5	30	15	17	148	0.24	0.16-0.32	3.3	3.0- 3.6
2-hour.....	5	30	15	17	145	0.06	0.04-0.10	1.3	1.0- 1.4
4-hour.....	5	30	15	17	118	0.01	0.01-0.02	0.5	0.5- 0.5
8-hour.....	5	30	15	17	152	0.01	0.01-0.01	0.4	0.0- 0.6
24-hour.....	5	30	15	17	155	0.00	0.00-0.00	0.5	0.5- 0.6

¹ Each mean value represents the average of three analyses.

of quinaldine residue of $0.29\text{ }\mu\text{g/g}$ and a mean concentration of MS-222 residue of $5.2\text{ }\mu\text{g/g}$ at 0-hour withdrawal. After 4 hours of withdrawal the MS-222 residue approached the background of the controls and the quinaldine residue had decreased to a mean concentration of $0.02\text{ }\mu\text{g/g}$.

Rainbow trout exposed at 12°C to a mixture of 10 mg of quinaldine sulfate and 40 mg of MS-222 per liter of water for 5.5 minutes contained a mean concentration of quinaldine residue of $0.72\text{ }\mu\text{g/g}$ and a mean concentration of MS-222 residue of $11.9\text{ }\mu\text{g/g}$ at the 0-hour withdrawal. After 8 hours of withdrawal the MS-222 residue was equal to the background of the controls ($0.5\text{ }\mu\text{g/g}$); however, the 24-hour withdrawal samples showed a residue of $1.0\text{ }\mu\text{g/g}$ of MS-222. The 24-hour withdrawal samples showed no quinaldine residue. Those exposed at 12°C to a mixture of 10 mg of quinaldine sulfate and 40 mg of MS-222 per liter of water for 15 minutes contained a mean concentration of quinaldine residue of $1.44\text{ }\mu\text{g/g}$ and a mean concentration of MS-222 residue of $17.0\text{ }\mu\text{g/g}$. After 24 hours of withdrawal the MS-222 residue (including background aromatic amines) was still slightly higher ($1.0\text{ }\mu\text{g/g}$) than the background of the controls ($0.5\text{ }\mu\text{g/g}$). The 24-hour withdrawals contained no detectable quinaldine residue.

Rainbow trout exposed at 17°C to a mixture of 5 mg of quinaldine sulfate and 30 mg of MS-222 per liter of water for 5.5 minutes contained a mean concentration of quinaldine residue of $0.53\text{ }\mu\text{g/g}$ and a mean concentration of MS-222 residue of $6.9\text{ }\mu\text{g/g}$ at the 0-hour withdrawal. After 4 hours of withdrawal the MS-222 residue had decreased to within the range of the background in the controls, and the mean concentration of quinaldine residue had decreased to $0.03\text{ }\mu\text{g/g}$. Rainbow trout exposed at 17°C to a mixture of 5 mg of quinaldine sulfate and 30 mg of MS-222 per liter of water for 15 minutes contained a mean concentration of quinaldine residue of $1.05\text{ }\mu\text{g/g}$ and a mean concentration of MS-222 residue of $16.5\text{ }\mu\text{g/g}$. After 24 hours of withdrawal the MS-222 residue had decreased to the background of the controls and no quinaldine residue was detected.

Lake trout

Hatchery-reared lake trout were exposed to a mixture of 10 mg of quinaldine sulfate and 40 mg of MS-222 per liter of water at 12°C for 5.5 and 15 minutes (table 4). Those exposed to this combination for 5.5 minutes contained mean concentrations of residues of $0.74\text{ }\mu\text{g/g}$ and $14.1\text{ }\mu\text{g/g}$ of quinaldine and MS-222, respectively. After 24 hours of withdrawal the MS-222 residue had decreased to less than the background of the controls and no quinaldine residue was detected. Those exposed to this combination for 15 minutes contained mean concentrations of residues of $1.26\text{ }\mu\text{g/g}$ and $17.9\text{ }\mu\text{g/g}$ of quinaldine and MS-222, respectively. The 24-hour withdrawal samples showed no residue above the background of the control.

Brook trout

Hatchery-reared brook trout were exposed at 9°C to a mixture of 10 mg of quinaldine sulfate and 40 mg of MS-222 for 5.5 minutes (table 4). Two weight groups were tested. Brook trout weighing approximately 0.3 kg contained mean concentrations of residues of $1.17\text{ }\mu\text{g/g}$ and $9.2\text{ }\mu\text{g/g}$ of quinaldine and MS-222, respectively; those weighing approximately 0.8 kg contained $0.96\text{ }\mu\text{g/g}$ and $6.4\text{ }\mu\text{g/g}$ of quinaldine and MS-222, respectively. Both groups showed no detectable residue above the background of the controls of either anesthetic after the last withdrawal interval.

Northern pike

Spawning adult northern pike from the Mississippi River were exposed to a mixture of 20 mg of quinaldine sulfate and 50 mg of MS-222 per liter of water at 7°C and to a mixture of 20 mg of quinaldine sulfate and 75 mg of MS-222 per liter of water at 12°C for 30 minutes (table 5). The fish treated at 7°C contained mean concentrations of $1.60\text{ }\mu\text{g/g}$ and $9.6\text{ }\mu\text{g/g}$ residues of quinaldine and MS-222, respectively. After 24 hours of withdrawal no residue of quinaldine or MS-222 was detected in this group. Those treated at 12°C contained a mean concentration of quinaldine residue of $1.80\text{ }\mu\text{g/g}$ at the 0-hour withdrawal and no quinaldine residue was detected after 24 hours of

Table 4.--Residues of anesthetics in muscle tissue of lake trout treated with mixtures of quinaldine sulfate (QdSO_4) and MS-222 at 12° C for 5.5 and 15 minutes and brook trout treated with mixtures of quinaldine sulfate (QdSO_4) and MS-222 at 9° C for 5.5 minutes

Withdrawal interval	Treatment			Mean weight (kg)	Residues (µg/g) ¹					
	QdSO ₄ (mg/l)	MS-222 (mg/l)	Exposure (min)		Quinaldine		MS-222			
					Mean	Range	Mean	Range		
Lake trout										
Control.....	0	0	0	1.63	0.00	0.00-0.00	0.5	0.5-	0.6	
0-hour.....	10	40	5.5	1.40	0.74	0.55-1.02	14.1	10.0-	17.4	
1-hour.....	10	40	5.5	1.47	0.24	0.19-0.27	1.0	1.0-	1.0	
4-hour.....	10	40	5.5	1.27	0.02	0.02-0.03	0.5	0.5-	0.5	
8-hour.....	10	40	5.5	1.78	0.01	0.00-0.02	0.0	0.0-	0.0	
24-hour.....	10	40	5.5	1.35	0.00	0.00-0.00	0.2	0.0-	0.5	
0-hour.....	10	40	15	1.82	1.26	1.05-1.60	17.9	14.8-	24.0	
8-hour.....	10	40	15	1.25	0.01	0.00-0.01	0.5	0.5-	0.5	
24-hour.....	10	40	15	1.57	0.00	0.00-0.00	0.2	0.0-	0.5	
Brook trout										
Control.....	0	0	0	0.30	0.00	0.00-0.00	0.6	0.5-	1.0	
0-hour.....	10	40	5.5	0.30	1.17	0.71-1.80	9.2	7.0-	13.4	
1-hour.....	10	40	5.5	0.31	0.20	0.17-0.24	1.2	0.6-	2.0	
2-hour.....	10	40	5.5	0.33	0.03	0.02-0.04	0.9	0.5-	1.4	
4-hour.....	10	40	5.5	0.30	0.02	0.02-0.02	0.9	0.5-	1.6	
8-hour.....	10	40	5.5	0.31	0.01	0.01-0.01	0.5	0.5-	0.6	
24-hour.....	10	40	5.5	0.30	0.00	0.00-0.00	0.6	0.5-	1.0	
Control.....	0	0	0	0.80	0.00	0.00-0.01	0.5	0.5-	0.5	
0-hour.....	10	40	5.5	0.88	0.96	0.46-1.31	6.4	3.6-	10.4	
15-hour.....	10	40	5.5	0.68	0.00	0.00-0.00	0.5	0.5-	0.5	

¹ Each mean value represents the average of three analyses.

Table 5.--Residues of anesthetics in muscle tissue of northern pike treated with mixtures of quinaldine sulfate (QdSO_4) and MS-222 for 30 minutes at 7° and 12° C and in muscle tissue of channel catfish treated with the combination anesthetic for 30 minutes at 19° C

Withdrawal interval	Treatment			Mean weight (kg)	Residues (μg/g) ¹				
	QdSO ₄ (mg/l)	MS-222 (mg/l)	Temp. (°C)		Quinaldine		MS-222		
					Mean	Range	Mean	Range	
Northern pike									
Control.....	0	0	7	1.43	0.00	0.00-0.00	0.6	0.5-	0.6
0-hour.....	20	50	7	1.21	1.60	1.40-1.70	9.6	8.0-	12.0
1-hour.....	20	50	7	1.02	0.53	0.48-0.56	2.8	2.8-	2.8
2-hour.....	20	50	7	1.13	0.33	0.22-0.42	1.9	1.6-	2.0
4-hour.....	20	50	7	1.20	0.06	0.05-0.08	1.0	1.0-	1.0
24-hour.....	20	50	7	1.21	0.00	0.00-0.00	0.6	0.6-	0.6
Control.....	0	0	12	1.48	0.00	0.00-0.00	0.6	0.5-	0.6
0-hour.....	20	75	12	1.67	1.80	1.50-1.90	Lost samples		
1-hour.....	20	75	12	2.08	0.34	0.24-0.48	3.0	2.6-	3.6
2-hour.....	20	75	12	1.53	0.07	0.06-0.08	1.4	1.4-	1.4
4-hour.....	20	75	12	1.55	0.04	0.02-0.05	1.0	1.0-	1.0
6-hour.....	20	75	12	1.94	0.02	0.01-0.03	0.5	0.5-	0.6
24-hour.....	20	75	12	1.65	0.00	0.00-0.00	Lost samples		
Channel catfish									
Control.....	0	0	19	1.27	0.00	0.00-0.00	0.3	0.2-	0.6
0-hour.....	40	60	19	1.50	6.92	6.37-7.31	13.7	11.4-	15.2
1-hour.....	40	60	19	1.27	3.19	2.96-3.39	4.9	4.2-	6.0
2-hour.....	40	60	19	1.36	1.79	1.52-2.18	3.6	2.8-	5.0
4-hour.....	40	60	19	1.27	0.56	0.44-0.74	0.6	0.2-	1.4
6-hour.....	40	60	19	1.32	0.30	0.10-0.51	0.4	0.1-	1.0
24-hour.....	40	60	19	1.14	0.00	0.00-0.01	0.1	0.0-	0.1

¹ Each mean value represents the average of three analyses.

withdrawal. The 0-hour and 24-hour withdrawal samples of this group for MS-222 analysis were lost; however, the 1-hour withdrawal sample contained a mean MS-222 residue of $3.0 \mu\text{g/g}$ which is very close to the concentration of MS-222 residue found in the 1-hour withdrawal samples treated at 7°C ($2.8 \mu\text{g/g}$). The residue of MS-222 in the 6-hour withdrawal samples was within the background of the controls.

Channel catfish

Hatchery-reared channel catfish were exposed to a mixture of 40 mg of quinaldine sulfate and 60 mg of MS-222 per liter of water at 19°C for 30 minutes (table 5). They contained mean concentrations of residues of $6.92 \mu\text{g/g}$ and $13.7 \mu\text{g/g}$ of quinaldine and MS-222, respectively. After 24 hours of withdrawal the MS-222 residue was less than the background of the controls and no quinaldine residue was detected.

Largemouth bass

Hatchery-reared largemouth bass were exposed to a mixture of 20 mg of quinaldine sulfate and 40 mg of MS-222 per liter of water at 19°C for 30 minutes (table 6). The mean concentrations of residues of quinaldine and MS-222 at 0-hour were $4.07 \mu\text{g/g}$ and $15.1 \mu\text{g/g}$, respectively. After 24 hours of withdrawal no residues of quinaldine or MS-222 were detected.

Bluegill

Hatchery-reared bluegills were exposed to a mixture of 10 mg of quinaldine sulfate and 40 mg of MS-222 per liter of water at 19°C for 30 minutes (table 6). The mean concentrations of residues of quinaldine and MS-222 at 0-hour were $3.13 \mu\text{g/g}$ and $27.3 \mu\text{g/g}$, respectively. After 24 hours of withdrawal no residues of quinaldine or MS-222 were detected.

Walleye

Spawning adult walleyes from the Mississippi River were exposed to a mixture of 10 mg of quinaldine sulfate and 30 mg of MS-222 per liter of water at 7°C for 30 minutes (table 6). The mean concentrations of residues of quinaldine and MS-222 at 0-hour were 2.20

$\mu\text{g/g}$ and $14.1 \mu\text{g/g}$, respectively. After 6 hours of withdrawal from the mixture, the quinaldine residue had decreased to a mean of $0.27 \mu\text{g/g}$ and the MS-222 residue had decreased to $2.3 \mu\text{g/g}$. Only enough fish were available for 6 hours of withdrawal.

DISCUSSION

The decrease in concentration of quinaldine and MS-222 residues during withdrawal of the fish from the mixed anesthetic follow a pattern similar to that of the individual anesthetics (Walker and Schoettger, 1967; Schoettger et al., 1967; Allen et al., 1972; Sills and Harman, 1970; and Sills et al., 1973). After 24 hours of withdrawal residues of both anesthetics decreased to near the background reading of the controls for MS-222 and to less than $0.01 \mu\text{g/g}$ for quinaldine.

More residues of the two anesthetics were accumulated in smaller brook trout (0.3 kg) than in larger fish (0.8 kg). At the 0-hour withdrawal period 1.22 times more quinaldine residue and 1.43 times more MS-222 were found in the smaller fish than in the larger fish.

MS-222 appears to be taken up by both coldwater and warmwater fish more readily than quinaldine. The mixed anesthetic solutions contained from 1.5 to 6 times higher concentrations of MS-222 than quinaldine sulfate and muscle residues at the 0-hour withdrawal interval contained from 2.0 to 23 times higher concentrations of MS-222 than quinaldine residue.

The warmwater species were exposed to the highest concentrations of the anesthetics at the highest temperature. This group of fish showed slightly higher concentrations of anesthetic residues at the 0-hour withdrawal than the coldwater fish.

The length of exposure influenced the concentration of anesthetic residues as found by the earlier investigators. Fish exposed to the same concentrations of the mixture of anesthetics for 15 minutes contained from 1.2 to 3.4 times the concentration of quinaldine residue and from 1.3 to 2.4 times the MS-222 residue as those exposed for 5.5 minutes, with the exception of coho salmon which showed higher MS-222 residues in the 5.5-minute exposure.

Table 6.--Residues of anesthetics in muscle tissue of largemouth bass and bluegills treated with mixtures of quinaldine sulfate (QdSO₄) and MS-222 for 30 minutes at 19°C and walleyes treated with the combination anesthetic for 30 minutes at 7°C

Withdrawal interval	Treatment		Mean weight (kg)	Residues (μg/g) ¹			
	QdSO ₄ (mg/l)	MS-222 (mg/l)		Quinaldine		MS-222	
				Mean	Range	Mean	Range
Largemouth bass							
Control	0	0	1.09	0.00	0.00-0.00	0.3	0.2- 0.6
0-hour	20	40	1.18	4.07	3.10-4.60	15.1	10.4-19.6
1-hour	20	40	1.04	0.56	0.46-0.62	2.7	1.6- 4.0
2-hour	20	40	1.09	0.49	0.43-0.54	2.6	2.4- 3.0
4-hour	20	40	1.22	0.17	0.14-0.22	0.6	0.1- 1.6
6-hour	20	40	0.91	0.06	0.06-0.07	0.9	0.6- 1.0
24-hour	20	40	1.27	0.00	0.00-0.00	0.2	0.2- 0.2
Bluegill							
Control	0	0	0.12	0.00	0.00-0.00	0.3	0.1- 0.6
0-hour	10	40	0.14	3.13	2.80-3.80	27.3	24.0-32.0
1-hour	10	40	0.15	0.30	0.27-0.34	3.5	2.8- 4.8
2-hour	10	40	0.14	0.14	0.12-0.17	1.5	1.4- 1.6
4-hour	10	40	0.15	0.02	0.02-0.03	0.5	0.2- 0.6
6-hour	10	40	0.17	0.02	0.01-0.04	0.3	0.2- 0.6
24-hour	10	40	0.15	0.00	0.00-0.00	0.1	0.0- 0.1
Walleye							
Control	0	0	0.81	0.00	0.00-0.00	0.7	0.6- 1.0
0-hour	10	30	0.89	2.20	2.00-2.40	14.1	13.6-14.6
1-hour	10	30	1.14	0.86	0.64-1.10	4.6	3.0- 6.2
2-hour	10	30	0.76	0.38	0.17-0.64	4.0	2.8- 5.0
6-hour	10	30	1.02	0.27	0.04-0.53	2.3	1.4- 2.6

¹ Each mean value represents the average of three analyses.

CONCLUSIONS

1. The residues of quinaldine and MS-222 in the species tested varied considerably depending on concentration of the anesthetic, temperature, and length of exposure. As any of these parameters was increased, the residue concentrations at 0-hour withdrawal increased.
2. The residue concentrations of quinaldine decreased to less than 0.01 $\mu\text{g/g}$ and those of MS-222 decreased to near the range of the background of the controls after 24 hours of withdrawal.
3. MS-222 is taken up more readily from the mixed anesthetic solutions than quinaldine.

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INVESTIGATIONS IN FISH CONTROL

- 56. Toxicity of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) to 10 Species of Algae**
- 57. Acute Toxicities of 3-Trifluoromethyl-4-nitrophenol (TFM) and 2', 5-Dichloro-4'-nitrosalicylanilide (Bayer 73) to Larvae of the Midge Chironomus tentans**
- 58. Acute Toxicity of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) to Nymphs of Mayflies (Hexagenia sp.)**
- 59. Toxicity and Residue Dynamics of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) in Aquatic Invertebrates**



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FOREWORD

The lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), has been used extensively to control larvae of the sea lamprey (Petromyzon marinus) in the Great Lakes. While the toxicity of TFM to lamprey is well documented (Investigations in Fish Control, number 44), its effects and those of mixtures of TFM with Bayer 73 (2-aminoethanol salt of 2'5-dichloro-4'-nitrosalicylanilide) on other organisms are unknown. The toxicity of Bayer 73 to fish was reported in IFC, number 19.

A petition for the registration of TFM must include data describing its effects on the non-target biota and on its fate within treated animals and in the environment. Studies were conducted at the Fish Control Laboratory, La Crosse, Wisconsin and under contract by researchers at other laboratories to develop the necessary data.

The following papers concern the effects of TFM on selected species of algae and of TFM, Bayer 73, and/or mixtures of the two on selected invertebrates under laboratory conditions. These papers represent the first of a continuing series to be published in Investigations In Fish Control. All will be used to support petitions for registration to permit the continued use of lampricides. Subsequent papers in the IFC series will concern the toxicity of TFM to fishes, invertebrates, and macrophytes, the efficacy of various TFM formulations, the residue patterns associated with applications, and the biotransformation of TFM in fish and invertebrates.

Fred P. Meyer, Director
Fish Control Laboratories

56. Toxicity of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) to 10 Species of Algae

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TOXICITY OF THE LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) TO 10 SPECIES OF ALGAE

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ABSTRACT

The toxicity of analytical and field grades of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to unialgal cultures of four green algae, four blue-green algae, and two species of diatoms was examined in 96-h toxicity tests. Growth was measured by daily optical density readings, cell counts of nonfilamentous species, and a gravimetric determination of maximum standing crop at the end of the tests. A 50% inhibition of growth (calculated from optical density data) occurred at concentrations less than 10 mg/l of TFM for all species tested except *Chlorella pyrenoidosa*, which was resistant at concentrations greater than 15 mg/l. Inhibition of growth was lowest in the blue-green species (50% effect levels, 9-10 mg/l), generally intermediate in the green algae, and severe in diatoms (50% effect levels, 1-4 mg/l). Field grade TFM tended to be more toxic than the analytical grade. Similar toxicity relationships were indicated by each of the three types of measurements made.

Growth tests conducted with high concentrations of TFM and subsequent filtration of the cells and resuspension in toxicant-free medium indicated that exposure to TFM at concentrations of 30 mg/l for 96 h did not destroy the viability of algal cells but temporarily inhibited growth.

INTRODUCTION

The toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to representatives of most animal groups has been examined in several investigations (see Schnick 1972 for a review), but knowledge of the effects of the toxicant on nontarget plant and algal species is limited (Howell 1966; Haas 1970). The compound is known to be phytotoxic and has been used at relatively high concentrations for the control of rooted aquatic plants (Josephs 1961). It is necessary to determine the effects of TFM on a variety of algal species, for it is well understood that any changes in these primary producers will influence each successive trophic level in the aquatic community.

This investigation was designed to determine the 96-h toxicity of analytical and field grade TFM to unialgal cultures and to determine the effects of TFM on growth and production of selected diatoms and green and blue-green algae. Growth was measured by making daily optical density readings and cell counts of nonfilamentous species, and determining maximum specific growth rates and total biomass produced by each culture.

METHODS

Unialgal cultures of four green algae, four blue-green algae, and two species of diatoms were obtained from the sources listed in Table 1. Several culture media were evaluated

Table 1. Scientific names and sources of algal species

Species	Source
Chlorophyta	
<u>Scenedesmus quadricauda</u> (Turp.) Breb.	Indiana Culture Collection, Indiana University
<u>Stigeoclonium tenue</u> Kutz.	Indiana Culture Collection, Indiana University
<u>Selenastrum capricornutum</u> Printz	National Eutrophication Research Program-EPA
<u>Chlorella pyrenoidosa</u> Chick	Indiana Culture Collection, Indiana University
Cyanophyta	
<u>Cylindrospermum</u> sp.	Indiana Culture Collection, Indiana University
<u>Anabaena flos-aquae</u> (Lyngb.) De Brebisson	National Eutrophication Research Program-EPA
<u>Nostoc linckia</u> (Roth) Born. et Flash.	Michigan State University Culture Collection
<u>Anabaena cylindrica</u> Lemmerman	Michigan State University Culture Collection
Chrysophyta	
<u>Nitzschia</u> sp.	Indiana Culture Collection, Indiana University
<u>Navicula pelliculosa</u> (Breb.) Hilse	Indiana Culture Collection, Indiana University

and the three that produced best growth of the three algal groups were used: Green algae were grown in ASM-1 medium (Gorham et al. 1964; also in Eberly 1967), blue-green algae in Allen and Arnon medium (Allen and Arnon 1955), and diatoms in the National Eutrophication Research Program medium

(Environmental Protection Agency [EPA] 1971) with the addition of silica (10 mg/l). The chemical composition of each medium is shown in Table 2. The pH of each medium was adjusted to 7.5 ± 0.2 by the addition of 0.1N HCl or 0.1N NaOH. Stock cultures were transferred to fresh media weekly to insure a

Table 2. Chemical composition of synthetic algal nutrient media

Element	Concentration in culture (mg/l -major; μg/l -minor)		
	NERP ^a with silica	Allen and Arnon (1955)	ASM-1
<u>Major</u>			
N	4.200	0.270	4.753
P	0.186	7.790	6.201
Mg	2.904	3.081	7.080
S	1.911	4.353	4.742
C	2.143	1.155	2.384
Ca	1.202	2.559	8.073
Na	11.001	12.735	76.970
K	0.469	20.735	7.812
Cl	6.611	23.585	96.465
Si	10.00	--	--
<u>Minor</u>			
B	32.5	62.5	431.0
Mn	115.374	62.4	333.0
Zn	15.691	6.3	210.0
Co	0.354	1.2	3.71
Cu	0.004	2.5	0.372
Mo	2.878	15.0	67.0
Fe	33.051	483.0	188.0
V	--	1.30	--

^a National Eutrophication Research Program.

continuous supply of cells in logarithmic growth phase to serve as inoculum for the toxicity tests.

Procedures and statistical analysis of the data followed the basic recommendations of the National Eutrophication Research Program (EPA 1971). All toxicity tests were carried out in a walk-in environmental chamber where incubation conditions were constant throughout the experiments. Cultures were maintained at 23 ± 1 C under continuous, cool-white fluorescent lighting of 400 ($\pm 10\%$) foot-candles. The inoculum for each test was adjusted to yield an initial cell concentration of about 1.0×10^4 cells/ml. Tests were conducted in 250-ml polyurethane-stoppered Erlenmeyer flasks with 60 ml of autoclaved medium on reciprocating shakers operating at 80 oscillations per minute.

Preliminary tests to define the range of toxicity for several species established the 50% effect at 5 to 10 mg/l; therefore, concentrations of 2.5, 5.0, 7.0, 8.0, 10.0, and 15.0 mg/l and a control were used for each toxicity test. Field grade and analytical grade TFM were tested simultaneously, with three replicates for each toxicant concentration; thus 42 culture flasks were used for each test. All data presented represent an average of two or three of these toxicity tests.

Purified TFM(A) (95% Aldrich lot #060217) and field grade (#1414, 35.7%, Drum 279 of Batch 6) TFM were used in all tests. Concentrations were based on active TFM rather than on the formulation. Primary and secondary stock solutions of the toxicant were made up in acetone and water, respectively, and periodically renewed throughout the experiments. All test flasks received 7.8 mg acetone per 60 ml culture (0.130 gm/l) from the stock solutions. Preliminary tests with three times this acetone concentration produced no measurable effects on algal growth.

Each toxicity test was conducted for 96 h to establish the lethal concentrations. The growth of the algae was determined spectrophotometrically at 680 nm on a Beckman DB spectrophotometer or a Bausch and Lomb Spectronic 20. The unicellular forms were counted

directly under a microscope with a hemacytometer.

The specific growth rate as defined by the following formula was calculated for all concentrations of each toxicity test.

$$u = \frac{\ln (X_2/X_1)}{t_2 - t_1} \text{ days}^{-1}$$

where X_2 = biomass concentration at end of selected time interval

X_1 = biomass concentration at beginning of selected time interval

$t_2 - t_1$ = elapsed time in days between selected determinations of biomass (EPA 1971).

We calculated linear regressions of the effects of both analytical and field grade TFM on maximum standing crop of algae, employing the following function:

$$\text{dry weight, } y = a + b (\text{TFM concentration})$$

Biomass was expressed in milligrams dry weight of each culture.

Maximum standing crop was determined gravimetrically for each test flask at the end of the 96-h toxicity test by filtering a measured portion of algal suspension through a tared Millipore® filter type AA with 0.80- μ m pores. Filters were oven-dried at 80 C for 24 h, cooled in a desiccator, and weighed. A correction factor was developed and subtracted to correct for loss of weight of the filters during washing. Toxicant concentration was checked at the beginning and end of each toxicity test by comparison with a standard curve on a Klett-Summerson colorimeter. Concentrations of TFM in the test solutions and pH at the termination of the test were checked on a portion of the medium filtered free of algal cells.

Slight decreases in TFM concentration were observed at the termination of each test, presumably due to absorption and uptake of TFM

by algal cells. Changes in pH between initiation and termination of the test were of the order of 0.1–0.4 pH units and were not consistently different between media.

The optical density data were analyzed by the methods of Litchfield and Wilcoxon (1949) for the evaluation of median effective concentrations (EC50) and establishment of 95% confidence limits. The EC50 is the TFM concentration that causes a 50% inhibition of algal growth when compared with control cultures growing simultaneously in the absence of the lampricide.

RESULTS

Toxicity Tests

The inhibition of growth by TFM differed for the different algal taxa (Table 3; Fig. 1). In general, it was lowest for the blue-green algae, intermediate for the green algae, and most severe for diatoms (Fig. 2). Exceptions were the unicellular green alga Chlorella pyrenoidosa, which appeared to be very resistant to TFM (EC50 greater than 15 mg/l of TFM) and the filamentous blue-green algae Anabaena flos-aquae and Anabaena cylindrica, which were highly susceptible to the lampricide (EC50's, 1.8 to 4.7 mg/l of TFM). Although the EC50 values were slightly lower for field grade than for analytical TFM for each species tested, the differences were statistically significant only for the highly sensitive diatom Nitzschia sp. However, the data suggest that the additive N, N-dimethylformamide in field grade material may augment the toxicity of the compound.

Specific Growth Rates

The maximum specific growth rate (μ_{max} --see Table 4) for individual species and concentrations at any time during incubation occurred on the second or third day of each toxicity test.

The values reflect the toxicity data presented in Table 3; the growth rates were generally best in control cultures and gradually

declined as TFM concentration increased. Growth rates were more severely depressed in cultures with field grade than with analytical grade TFM. Negative growth rates for the diatoms Nitzschia sp. and Navicula pelliculosa indicate mortality of the inoculum at the higher dose levels on the second day of exposure.

In several of the cultures in 2.5 mg/l of analytical grade TFM, growth was apparently stimulated initially, since the μ_{max} exceeded that of the controls. However, the cell density and standing crop at the end of the 4-day toxicity test were lower than in the controls, indicating that growth was suppressed at these low concentrations.

Regression analysis of toxicant concentration and maximum standing crop

The maximum standing crop for each test is defined as the weight of the total algal biomass at the end of the 4-day toxicity test, after filtration through a tared Millipore filter and oven-drying at 80 C for 24 h. Linear regressions of the effects of analytical and field grade TFM on maximum standing crop of algae were produced for the species whose growth was not severely limited by relatively low TFM concentrations.

The equations serve to establish a degree of linearity of response to the proportional increases in toxicant concentration (EPA 1971).

The linear relation between TFM concentration and maximum standing crop describes the effect of increasing TFM concentration, as evidenced by the relatively high regression coefficients for most species (Table 5). However, for species whose growth is severely limited by relatively low concentrations of the lampricide, a curvilinear relation may best describe the data. The gravimetric determination of growth (Table 5) is not as sensitive a measure of growth as is the optical density reading (Fig. 1)--for even though the optical density data indicate measurable growth, the gravimetric data do not.

Table 3. Growth inhibition of TFM^a to algae as measured by optical density

Species	96-h EC50 and 95% confidence interval (mg/l)	
	Field grade	Analytical grade
Chlorophyta		
<u>Scenedesmus quadricauda</u>	4.2 2.21-7.98	4.5 2.81-7.20
<u>Stigeoclonium tenue</u>	4.7 3.24-6.81	6.2 4.43-8.68
<u>Selenastrum capricornutum</u>	5.5 4.14-7.32	8.6 5.93-12.5
<u>Chlorella pyrenoidosa</u>	>15	>15
Cyanophyta		
<u>Cylindrospermum</u> sp.	9.0 7.50-10.8	9.9 3.96-24.8
<u>Anabaena flos-aquae</u>	3.6 2.40-5.40	4.2 2.47-7.14
<u>Nostoc linckia</u>	9.2 8.00-10.6	9.8 6.12-15.7
<u>Anabaena cylindrica</u>	1.8 0.82-3.96	4.7 2.90-7.61
Chrysophyta		
<u>Nitzschia</u> sp.	1.2 0.71-2.04	3.6 2.57-5.04
<u>Navicula pelliculosa</u>	3.2 2.00-5.12	5.2 3.71-7.28

^a Concentration based on active TFM.

The higher toxicity of the field formulations is again shown for all three groups of algae by the lower y-intercept and depressed regression of the total biomass produced in the field grade toxicity tests. The most sensitive species are distinguished by their high intercept values and relatively shallow slopes.

Cell Counts

A hemacytometer was employed for direct microscopic counting of Scenedesmus quadricauda, Selenastrum capricornutum, and Chlorella pyrenoidosa. Accurate counts of the other species were not possible. Filamentous

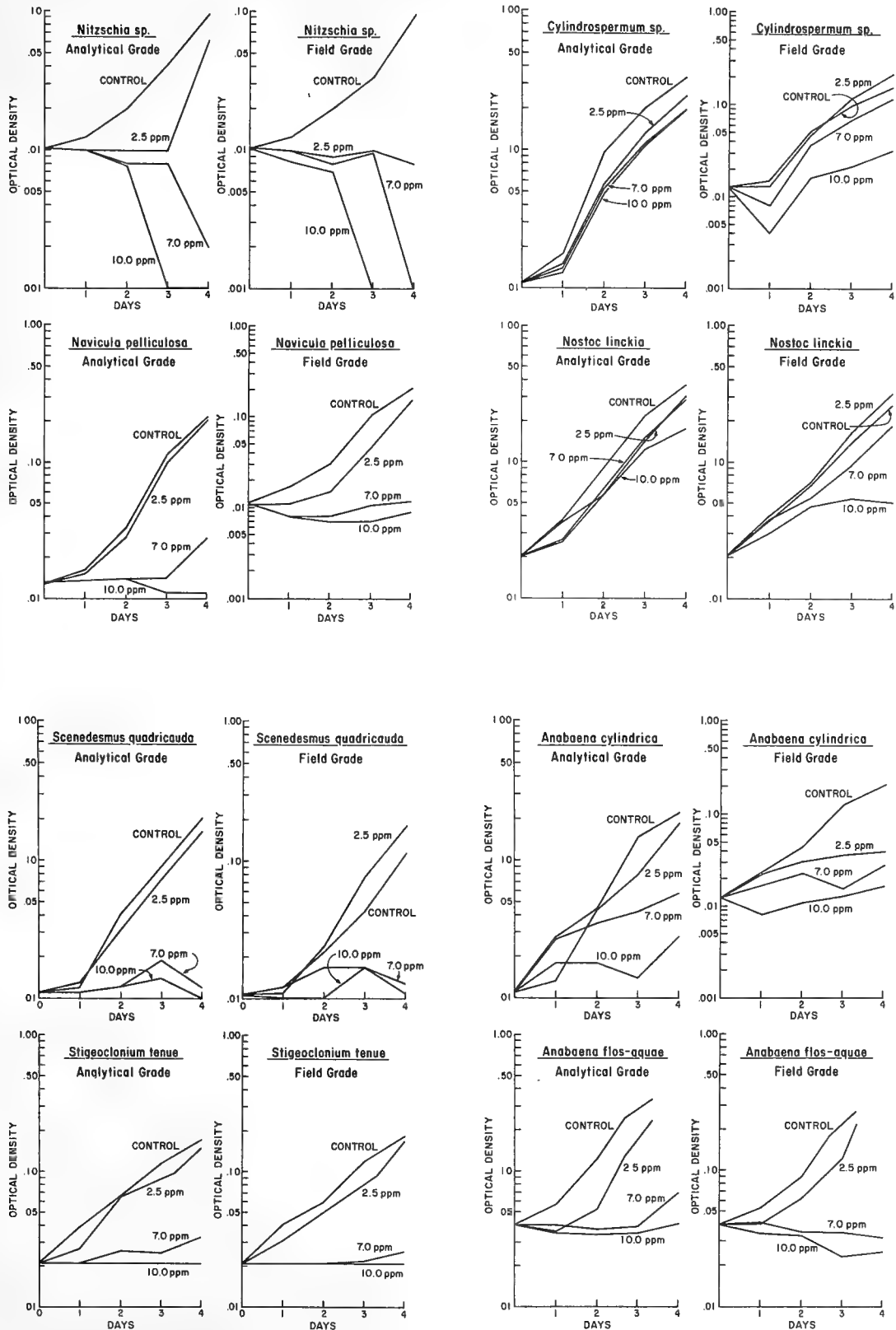


Fig. 1. Effects of analytical and field grade TFM on growth of algal cultures, measured as optical density.

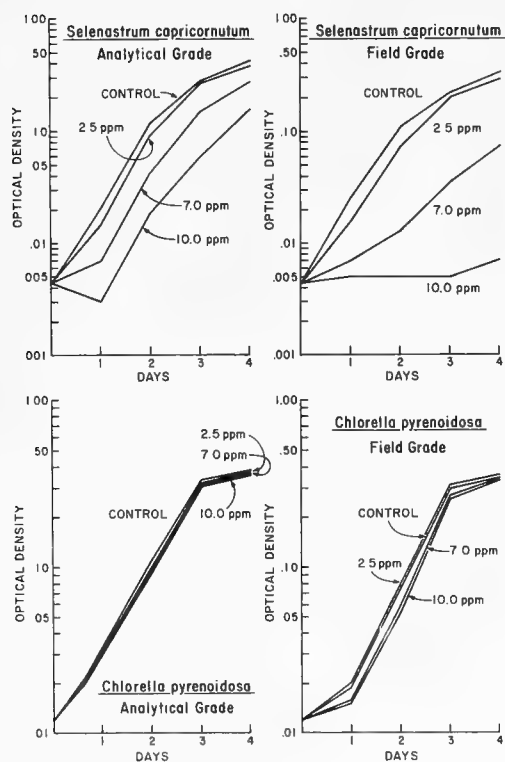


Fig. 1. Continued

species were difficult to count, and the diatoms tended to grow in small, tightly packed clumps. Attempts to equally disseminate the filamentous forms and clumping species by blending or dispelling them through a syringe were unsuccessful. The counts for the three species yielded an excellent representation of the inhibition of growth by TFM (Fig. 3).

Mortality Tests

Inasmuch as the toxicity tests showed a definite suppression of algal growth that was directly correlated with TFM concentration, we designed a series of experiments to determine whether the growth suppression was a result of (1) toxicant-induced mortality of algal cells, (2) a temporary growth suppression of the cells at the end of the 4-day toxicity test, or (3) a selective elimination of a proportion of the new cells being produced which gave the indication that no new growth was occurring. We established replicate toxicity tests using the standard technique and the TFM concentrations of 15 and 30 mg/l, which exceeded the 96-h lethal concentrations

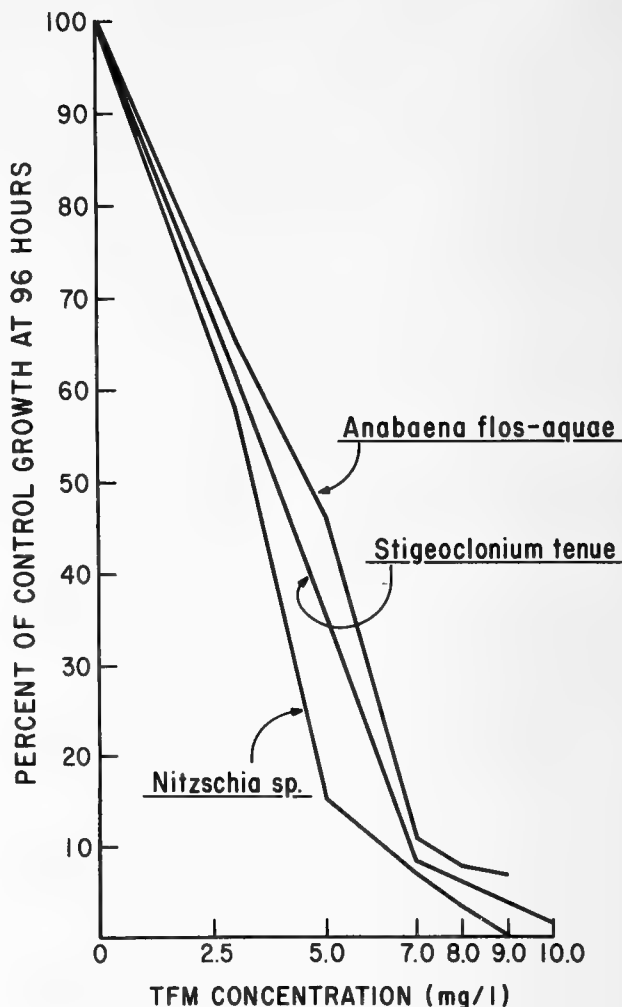


Fig. 2. Relative growth of representative algal species grown in increasing concentrations of analytical grade TFM, expressed as a percentage of control optical density at 96 h.

for all species except *Chlorella pyrenoidosa*. After the cells had been exposed at these concentrations for 96 h, they were filtered free of the dosed medium and resuspended in TFM-free medium. This procedure simulated a natural situation in which an algal population is exposed to high lampricide concentrations for a relatively long period (4 days) in a bay or backwater at the mouth of a stream being treated for lamprey control. No growth was observed in any of the cultures during the initial 96-h exposure to 30 mg/l, and *Chlorella pyrenoidosa* was the only species to produce measurable growth at 15 mg/l of TFM.

Table 4. Maximum specific growth rates (μ_{\max} --average of three replicates) of 10 algal species at different concentrations of TFM

Species, and grade of TFM	TFM Concentration (mg/l of active ingredient)						
	0	2.5	5.0	7.0	8.0	10.0	15.0
<u>Scenedesmus quadricauda</u>							
Analytical	1.230	0.867	0.815	0.351	0.231	0.239	0.201
Field	1.001	1.179	0.405	0.351	0.296	0.317	0.131
<u>Stigeoclonium tenue</u>							
Analytical	0.621	0.892	0.900	0.215	0.131	0.049	0.077
Field	0.718	0.476	0.270	0.166	0.140	0.049	0.000
<u>Selenastrum capricornutum</u>							
Analytical	1.751	1.825	1.959	1.839	1.546	1.405	1.497
Field	1.649	1.533	1.358	0.990	0.875	0.604	0.336
<u>Chorella pyrenoidosa</u>							
Analytical	1.619	1.445	1.351	1.440	1.834	1.488	1.660
Field	1.413	1.449	1.493	1.470	1.479	1.475	1.460
<u>Cylindrospermum sp.</u>							
Analytical	1.685	1.335	1.683	1.332	1.197	1.435	1.327
Field	1.224	1.264	1.163	0.486	0.836	0.767	0.692
<u>Anabaena flos-aquae</u>							
Analytical	0.784	0.900	0.667	0.582	0.412	0.095	0.093
Field	0.673	0.683	0.270	0.068	0.034	0.033	0.025
<u>Nostoc linckia</u>							
Analytical	0.811	0.747	0.723	0.658	0.593	0.631	0.525
Field	0.713	0.846	0.775	0.673	0.599	0.536	0.451
<u>Anabaena cylindrica</u>							
Analytical	1.212	1.241	0.728	0.315	0.515	0.195	0.221
Field	1.037	0.300	0.255	0.298	0.182	0.374	0.270
<u>Nitzschia sp.</u>							
Analytical	0.788	1.808	0.668	-0.930	-1.098	-	-
Field	1.037	0.104	0.104	-1.040	-1.099	-1.945	1.252
<u>Navicula pelliculosa</u>							
Analytical	1.229	1.253	1.184	0.693	0.095	-0.241	-0.105
Field	1.239	1.209	0.489	0.322	-0.134	0.255	0.104

Table 5. Maximum standing crop (dry weight in milligrams) of 10 species of algae after 96-h exposure to different TFM concentrations. Regression equations are given for species showing a linear response--Continued.

Species, and grade of TFM	TFM Concentration (mg/l of active ingredient)							Equation
	0	2.5	5.0	7.0	8.0	10.0	15.0	
<u>Chlorella pyrenoidosa</u>								
Analytical	5.6	5.2	5.4	5.2	5.4	5.0	5.0	$y = 5.467 - 0.037(X)$ $r^2 = .707$
Field	5.2	4.4	5.4	5.0	5.2	4.8	4.6	$y = 5.090 - 0.022(X)$ $r^2 = .806$
<u>Cylindrospermum</u> sp.								
Analytical	12.0	8.0	9.2	6.2	5.4	2.4	5.2	$y = 10.324 - 0.503(X)$ $r^2 = .635$
Field	5.4	6.8	5.0	3.6	4.0	1.6	0.8	$y = 6.535 - 0.391(X)$ $r^2 = .834$
<u>Anabaena flos-aquae</u>								
Analytical	10.3	8.8	6.8	2.2	1.4	1.4	1.4	$y = 10.100 - 0.942(X)$ $r^2 = .874$
Field	11.2	8.2	3.0	2.6	2.4	2.2	2.0	
<u>Nostoc linckia</u>								
Analytical	8.8	6.2	5.6	5.0	4.6	2.6	2.8	$y = 8.405 - 0.560(X)$ $r^2 = .922$
Field	5.6	6.4	5.6	2.6	2.4	0.4	0.0	$y = 7.129 - 0.648(X)$ $r^2 = .817$

Table 5. Maximum standing crop (dry weight in milligrams) of 10 species of algae after 96-h exposure to different TFM concentrations. Regression equations are given for species showing a linear response--Continued.

Species, and grade of TFM	TFM Concentration (mg/l of active ingredient)							Equation
	0	2.5	5.0	7.0	8.0	10.0	15.0	
<u>Anabaena cylindrica</u>								
Analytical	3.0	1.8	0.4	0.0	0.0	0.0	0.0	$y = 2.809 - 0.393(x)$ $r^2 = .951$
Field	3.8	0.0	0.0	0.0	0.0	0.0	0.0	
<u>Nitzschia</u> sp.								
Analytical	4.0	3.6	1.4	0.4	0.2	0.0	0.0	$y = 3.529 - 0.314(x)$ $r^2 = .760$
Field	4.2	0.2	0.2	0.2	0.0	0.0	0.0	
<u>Navicula pelliculosa</u>								
Analytical	8.4	6.6	5.0	1.0	0.0	0.0	0.0	$y = 7.341 - 0.640(x)$ $r^2 = .780$
Field	6.8	3.8	0.0	0.0	0.0	0.0	0.0	

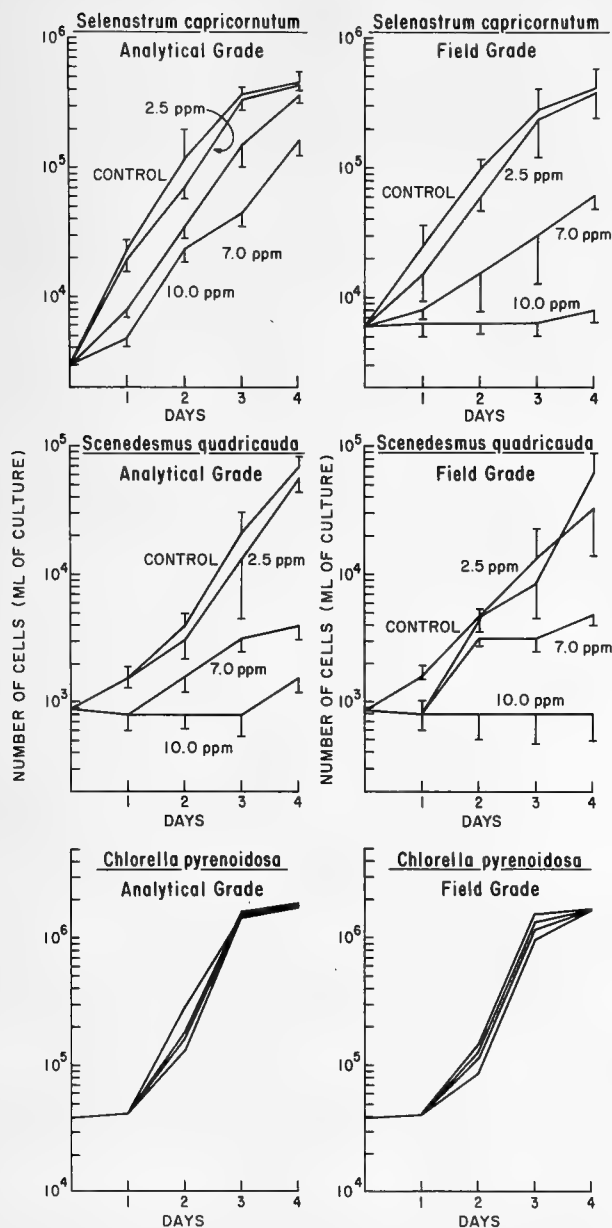


Fig. 3. Cell numbers in algal cultures grown in selected concentrations of analytical and field grade TFM.

After resuspension in toxicant-free medium, each alga showed little or no growth for 2 to 3 days. Soon after this lag, each culture grew, and cell density reached that in the controls after an average of 12 days, indicating that viability had not been destroyed at these high concentrations but that the ability to grow and reproduce was severely limited by the presence of the toxicant. The exception in this test was the sensitive diatom *Navicula pelliculosa*,

which did not grow after the 96-h exposure to 30 mg/l of TFM.

The remaining possibility--that the cells were indeed growing in the high concentrations, but that the toxicant was selectively eliminating a proportion of the new cells being produced--remains unresolved. However, microscopic examinations of TFM-exposed algal cells while they were being counted during the toxicity tests tended to indicate that no vegetative reproduction was occurring, and that TFM was producing severe sublethal effects on the integrity of the chloroplast and other cell inclusions. Misshaped and shrunken chloroplasts were commonly observed in cells exposed to the higher concentrations of TFM, but normal growth and reproduction ensued soon after the cells were resuspended in toxicant-free medium.

Another possibility that warranted further investigation was that the color imparted to the algal growth medium by release of the phenolate ion from TFM selectively shades out a portion of the light spectrum and thus inhibits growth of the cultures. To test this hypothesis, we added sufficient food coloring to the growth medium to produce a yellow-green color that absorbed at 475 Klett Units--an absorption similar to that of a 30 mg/l solution of analytical grade TFM. Both species tested--*Anabaena cylindrica* and *Selenastrum capricornutum*--produced greater cell densities in 96 h in the flask to which food coloring had been added than in the control flasks. Although the absorption spectra of TFM and food coloring may not be exactly equivalent, we assumed on the basis of this test that shading from the colors of the phenolate ion of TFM did not limit growth.

DISCUSSION

Tests of the toxicity of analytical (95%) and field grade (35.7%) TFM to unialgal cultures of representative green and blue-green algae and diatoms showed that field grade material tended to exert the greater toxicity, when expressed on the basis of percentage active ingredient; 96-h 50% effect values generally were 2 to 3 mg/l lower for field grade than

for analytical grade material. Although the values were not statistically different, the tendency agrees with tendencies demonstrated in insects and fish.

The 96-h 50% effect levels ranged from 1.2 to more than 15 mg/l for field grade TFM and from 3.6 to more than 15 mg/l for analytical grade material. The most pronounced effects of the toxicant were observed in the diatom cultures, where 50% tolerance limits ranged from 1.2 to 5.2 mg/l. Intermediary effects were observed among the green algae, where toxicity values generally ranged from 4 to 8 mg/l; the exception was Chlorella pyrenoidosa which was resistant to concentrations greater than 15 mg/l. Morgan (1972) found that a unicellular green alga, Chlamydomonas reinhardtii, grew well at 20 mg/l of Aroclor 1242, a concentration normally lethal to other aquatic life. Zweig et al. (1968) found Chlorella pyrenoidosa to be relatively resistant to low concentrations of the herbicides diquat and 1,4-benzoquinone; the effects of the herbicides were termed algistatic rather than algicidal.

We found the more TFM-resistant species among the blue-green algae although two species of Anabaena had EC50's which approached those of the sensitive diatom species. This high susceptibility of Anabaena is unexplained.

Lampricide sensitivity differed little between species; i.e., in all of them, progressively lower growth rates were correlated with progressively higher toxicant levels. Several of the species expressed a higher μ_{max} at 2.5 mg/l of TFM than did the controls, although the total biomass yield at the end of the 96-h test did not exceed the yield of control cultures. Stadnyk et al. (1971) reported similar increased growth rates and cell numbers, but not biomass, for cultures of Scenedesmus quadricauda exposed to 0.1 and 1.0 mg/l of organochlorine insecticides. The TFM may have been metabolized at low concentrations, either by the algal cells themselves or by contaminating bacteria. Although Klett Unit absorbance of filtered media did not change after any of the tests, the colorimeter

may have been insufficiently sensitive to detect metabolism of the lampricide.

The present study indicates that residues of TFM inhibited algal growth at concentrations that might be developed during stream treatments. The inhibition was only temporary, however, and even exposures to the high concentration of 30 mg/l for 4 days did not decrease the viability of algal cells when they were resuspended in toxicant-free water. Although metabolic activity such as oxygen evolution or carbon assimilation were not measured, an effect on these activities is implied in the total reproductive capacity of the algal cultures. Further experimentation may yield an understanding of the nature of the temporary growth inhibition.

CONCLUSIONS

1. The lampricide TFM inhibited growth of all algal species tested. Concentrations of less than 10 mg/l caused a 50% inhibition of growth in all species except Chlorella pyrenoidosa, which was resistant to test concentrations up to 15 mg/l.
2. Diatoms were the most sensitive species tested; 50% inhibition of growth occurred at concentrations of 1-4 mg/l of TFM. The green algae were intermediate in sensitivity. In blue-green algae, the least susceptible group, 50% inhibition of growth generally occurred near 10 mg/l of TFM.
3. Field grade TFM generally tended to be more toxic than analytical grade material.
4. TFM does not appear to be algicidal within the range of concentrations tested but rather produces a temporary algistatic inhibition of growth.

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**57. Acute Toxicities of 3-Trifluoromethyl-4-nitrophenol
(TFM) and 2',5-Dichloro-4'-nitrosalicylanilide
(Bayer 73) to Larvae of the Midge Chironomus tentans**

By Joseph A. Kawatski, Margaret M. Ledvina,
and Carl R. Hansen, Jr.



United States Department of the Interior
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ACUTE TOXICITIES OF 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) AND 2',5-DICHLORO-4'-NITROSALICYLANILIDE (BAYER 73) TO LARVAE OF THE MIDGE (CHIRONOMUS TENTANS)

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ABSTRACT

The toxicants 3-trifluoromethyl-4-nitrophenol (TFM) and 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73) were tested individually and together for toxicity to fourth instar Chironomus tentans in laboratory static tests at 22 ± 1 C. The 24-h EC₅₀ (immobility) values in soft water (total hardness, 40-48 mg/l as CaCO₃) were 1.55 mg/l of 95.7% TFM, 0.570 mg/l of 70% Bayer 73, and 0.658 mg/l of total toxicant for a 98:2 mixture of TFM and Bayer 73. As water hardness increased, toxicity of the materials individually and in mixtures decreased. Generally, the toxic effect of mixtures of TFM and Bayer 73 was additive; synergistic toxicity was apparent only in soft water at exposures of 24 to 72 h. Immobility was not coincident with death in chironomids. Considerably greater concentrations of toxicant were required to kill than to immobilize.

INTRODUCTION

The toxicant 3-trifluoromethyl-4-nitrophenol (TFM) has been used extensively and successfully to control the sea lamprey (Petromyzon marinus) in the Great Lakes. Authorization for the continued use of the chemical will depend on the outcome of current research designed to clarify the effects of TFM on a variety of nontarget organisms (Schnick 1972). The molluscicide Bayer 73 (2-aminoethanol salt of 2',5-dichloro-4'-nitrosalicylanilide) is applied with TFM to produce a 2% mixture, i.e., 98 parts of TFM (95.7%): 2 parts Bayer 73 (70% W.P.), here termed TFM:2B. This mixture has an apparent synergistic effect on lamprey larvae (Howell et al. 1964). Inasmuch as invertebrates constitute a vital portion of aquatic food webs, the elucidation of the toxicities of TFM and Bayer 73 to invertebrates is essential.

Among the invertebrates in most aquatic food chains, insect larvae, including midges, represent one of the most important links at a relatively low trophic level. We report here on the acute toxicities of TFM, Bayer 73, and

TFM-2B to laboratory-reared, fourth instar populations of Chironomus tentans Fabricius, a widely distributed benthic midge and an important fish food organism. For reference, we also determined the toxicity of antimycin A against this chironomid.

MATERIALS AND METHODS

The U.S. Fish and Wildlife Service, Fish Control Laboratory, La Crosse, Wisconsin, provided the following materials for this study: TFM (95.7% 3-trifluoromethyl-4-nitrophenol, Aldrich Chemical Co., Lot No. 060217); Bayer 73 (70% 2-aminoethanol salt of 2',5-dichloro-4'-nitrosalicylanilide, wettable powder, Chemagro Corp., Lot No. 8059410); antimycin A (95.5%, Ayerst Laboratories, Lot No. 1294-L); and reconstituted water. Soft, hard, and very hard waters, with respective total hardnesses of 40-48, 160-180, and 280-320 (as mg/l CaCO₃), were used in the toxicity tests. These waters were further characterized by Marking (1970). The food and substrate mixture consisted of ground Trainers Dog Rewards® (Horlick's Corp., Racine,

Wisconsin) and macerated paper hand towels (Crown Zellerback Corp., San Francisco, California). This mixture contained no detectable TFM or Bayer 73 residues as determined by gas-liquid chromatographic analysis.

Stock cultures of *C. tentans* were propagated in the laboratory in 5-, 20-, and 400-liter glass or fiberglass aquaria, in soft, reconstituted water at a temperature of 22 ± 1.5 C, under a 16-h photoperiod of mixed fluorescent and incandescent light. Food and substrate were added periodically to sustain growth and reproduction. Continuous gentle aeration was provided. This rearing system was essentially that of Derr and Zabik (1972).

About 48 h before each toxicity test, we transferred 10-20 organisms individually, with a curved blunt probe, into separate beakers containing 900 ml of well-aerated test water. The toxicants, dissolved in 50% acetone (in water), were then introduced into the exposure vessels to produce the appropriate test concentrations. The total amount of acetone added to each vessel and to control systems was 0.5 ml. The criterion for evaluating toxicant effect was immobility, noted after 8, 24, 48, 72, and 96 h of exposure. A test animal was considered immobile when it failed to move its body, jaws, or anal gills after it was touched lightly with a glass probe. The water was not aerated during exposures. Toxicity tests with TFM, Bayer 73, and TFM-2B were replicated at least three times for each of three water hardnesses at 22 ± 1 C. Using the Litchfield and Wilcoxon (1949) method, we calculated the EC50's from the combined data of at least three independent tests. (The EC50 is defined as the concentration of toxicant, based on total formulation weight, that immobilized 50% of the test organisms within the prescribed period of exposure.) Results of tests in which more than 20% of the control organisms died were discarded. From 114 to 590 animals were used to determine the EC50's for each chemical or combination of chemicals. The EC50's derived for TFM-2B were based on total toxicant, i.e., the sum of TFM and Bayer 73.

The toxicity of TFM-2B was compared with the toxicities of the two lampricides alone by

calculation of additive indices as described by L. L. Marking and V. K. Dawson (in preparation). The significance of index values is determined from ranges for the indices, using 95% confidence limits for the EC50 values. If the range for an index is above zero, the toxicity of the combined chemicals is synergistic; if the range is below zero, the toxicity is antagonistic.

RESULTS

Water quality had a direct effect on the toxicity of TFM, Bayer 73, and TFM-2B against *C. tentans* (Table 1.) Hard water with high pH (8.0-8.4) decreased the toxicity of the chemicals, whereas soft water with a lower pH (7.2-7.6) increased it. The influence of water quality was less pronounced with Bayer 73 than with TFM. In comparison with TFM, the toxicity of Bayer 73 to *C. tentans* was 2 to 3 times greater in soft water and 6 to 12 times greater in hard water.

At most water hardnesses and exposure periods, the addition of 2% of Bayer 73 reduced the amount of TFM required to immobilize chironomids. However, the ranges of additive indices for TFM-2B indicated that synergistic toxicity occurred only in soft water and then only at three intermediate exposure periods (Table 2). Additive index ranges overlapped zero in all water hardnesses at 8- and 96-h exposures; when test water was hard or very hard, the toxic effect of TFM-2B was additive.

Antimycin A was used as a reference chemical to determine the sensitivity of our laboratory population of *C. tentans*. In soft water, the 96-h LC50 and 95% confidence limits were determined to be 0.146 (0.095-0.224) $\mu\text{g}/\text{l}$.

DISCUSSION

Howell et al. (1964), who determined the lethal concentrations of TFM to lamprey larvae in water ranging from soft with a pH of 7.3 to very hard with a pH 8.5, reported that toxicity decreased as hardness and pH increased. They found, however, that even in hard water the amount of TFM-2B required to

Table 1. Toxicities of TFM, Bayer 73, and a 98:2 mixture of TFM and Bayer 73 to fourth instar Chironomus tentans in waters of different ranges of hardness at 22 ± 1 C

Chemical and water hardness (mg/l as CaCO_3)	EC50 (mg/l) ^a and 95% confidence intervals at:				
	8 h	24 h	48 h	72 h	96 h
TFM					
Soft (40-48)	1.65 1.45-1.88	1.55 1.32-1.83	0.968 0.815-1.15	0.790 0.631-0.989	0.534 0.415-0.688
Hard (160-180)	6.47 5.66-7.39	4.54 4.06-5.08	2.53 2.13-3.01	1.19 0.937-1.51	0.998 0.854-1.17
Very hard (280-320)	14.3 13.1-15.6	10.0 8.09-12.4	6.60 5.47-7.97	3.49 2.84-4.29	2.10 1.65-2.67
Bayer 73					
Soft (40-48)	0.744 0.550-0.998	0.570 0.458-0.710	0.367 0.289-0.467	0.347 0.257-0.469	0.228 0.171-0.304
Hard (160-180)	1.01 0.712-1.43	0.640 0.501-0.817	0.457 0.352-0.593	0.360 0.275-0.471	0.295 0.221-0.393
Very hard (280-320)	2.01 1.45-2.79	0.814 0.630-1.05	0.536 0.417-0.690	0.374 0.286-0.490	0.353 0.261-0.477
Mixture, TFM, and Bayer 73 (98:2)					
Soft (40-48)	1.38 1.18-1.61	0.658 0.474-0.913	0.400 0.312-0.513	0.358 0.283-0.453	0.328 0.262-0.416
Hard (160-180)	4.33 3.76-4.99	2.59 1.76-3.81	1.52 1.19-1.95	0.910 0.749-1.11	0.657 0.516-0.821
Very hard (280-320)	11.8 10.7-13.1	7.03 5.89-8.39	4.94 4.14-5.89	3.50 2.81-4.36	2.79 2.36-3.29

^a EC50 (immobility) values for mixture are expressed as total toxicant (TFM plus Bayer 73).

Table 2. Additive indices for the toxicity of a 98:2 mixture of TFM and Bayer 73 against Chironomus tentans in selected hardnesses at 22 ± 1 C

Water hardness (as mg/l CaCO_3)	Additive indices ^a and their ranges at:				
	8 h	24 h	48 h	72 h	96 h
Soft (40-48)	+0.167 -0.147-+0.566	+1.28 +0.930-+2.74	+1.34 +0.533-+2.58	+1.15 +0.354-+2.42	+0.585 -0.031-+1.56
Hard (160-180)	+0.348 -0.004-+0.814	+0.562 -0.072-+1.61	+0.526 -0.008-+1.34	+0.250 -0.242-+0.931	+0.450 -0.016-+1.18
Very hard (280-320)	+0.080 -0.161-+0.335	+0.161 -0.283-+0.731	+0.900 -0.338-+0.590	-0.170 -0.809-+0.322	-0.460 -1.21-+0.036

^a Indices with ranges less than zero suggest antagonism; indices with ranges that overlap zero suggest additive toxicity; and indices with ranges, greater than zero suggest synergism.

kill lampreys was less than the amount of TFM alone required to kill them. B. R. Smith (1967) also observed that the toxicity decreased as water hardness increased when Bayer 73 and TFM-2B were tested against lampreys and rainbow trout (*Salmo gairdneri*), and that the degree of synergism decreased as water hardness and pH increased. Our data and those of Kawatski (1973) show that the response of midges and ostracods is similar to that of fish.

Some chironomids that were immobilized by TFM and appeared dead revived when they were placed in toxicant-free water. Post-exposure observation enabled us to establish that, for TFM in soft water, the 8-h LC50 was 12 times greater than the immobility-based 8-h EC50. The disparity between EC50 and LC50 values resides with the difficulty in ascertaining whether a test animal is immobile, moribund, or dead. However, as exposure time lengthens, dead chironomids are more easily recognized, and the difference between the EC50's and LC50's in longer exposures (24-96 h) is much smaller.

Recovery of test organisms, especially after short-term exposure, was not surprising because earlier experiments (unpublished) in our laboratory showed that chironomids exposed to ¹⁴C-TFM rapidly biotransformed and excreted accumulated residue; this detoxication continued even after the organisms were immobilized. Because of these observations and the difficulty in defining death, particularly in small and/or normally sluggish invertebrates, it is clear to us that in short-term exposures LC50's can be accurately determined only if the affected organisms are held in toxicant-free water after the exposure period.

CONCLUSIONS

1. The toxicity of TFM, Bayer 73, and TFM-2B against *Chironomus tentans* decreases as water hardness and pH increase.
2. Generally, the toxic effect of TFM and Bayer 73 in combination (TFM-2B) is additive; synergistic toxicity occurs only in

soft water at exposure times of 24 to 72 hours.

3. Toxicant-induced immobility was not synonymous with death, as shown by the large percentage of toxicant-immobilized larvae that became active when they were placed in TFM-free water after exposure.

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**58. Acute Toxicity of the Lampricide
3-Trifluoromethyl-4-nitrophenol (TFM)
to Nymphs of Mayflies (Hexagenia sp.)**

By Calvin R. Fremling



United States Department of the Interior
Fish and Wildlife Service
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ACUTE TOXICITY OF THE LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) TO NYMPHS OF MAYFLIES (HEXAGENIA SP.)

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ABSTRACT

A recycling test apparatus and burrow-containing artificial substrates were used to determine the toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) against Hexagenia mayfly nymphs. Toxicity was relatively independent of temperature, but was greater in soft water than in hard water, and much greater at low than at high pH's; 12-h LC50's were 4.0 at pH 6.5 and 270.0 at pH 9.5.

INTRODUCTION

Hexagenia mayflies play a vital ecological role in lakes, rivers, and streams (Needham et al. 1935, Hunt 1953, Britt 1955, Fremling 1960, Swanson 1967). Nymphs of Hexagenia are important to fish because they convert organic detritus, algae, and bacteria into high quality fish food. The detritus-mayfly-fish food chain is short and efficient. Inasmuch as the life cycle of Hexagenia lasts at least a year in the Great Lakes and in most tributary streams, nymphs are available to fish in all seasons. Because the nymphs pass through many molts, sizes are available to suit the needs of most fish species.

Since Hexagenia nymphs prefer silt bottoms where they can construct burrows, they usually inhabit the same areas used by lamprey ammocoetes. Silted streams, for example, provide suitable habitat for both. It is important to determine the effect of lampricides on Hexagenia nymphs because they may be eradicated in lamprey control areas if the lampricides are toxic to them.

Hexagenia nymphs are good test organisms because they are easily collected and cultured and their large size makes them easy to handle and observe. Their tendency to abandon their burrows when stressed makes it possible to accurately assess early effects of toxicants.

METHODS

Either reconstituted water supplied by the Fish Control Laboratory, La Crosse, Wis., or water from a 12-m deep sand point well was used in the tests. The reconstituted water contained 48 mg/l of NaHCO_3 , 30 mg/l of CaSO_4 , 30 mg/l of MgSO_4 , and 2 mg/l of KCl; was slightly alkaline (pH 7.2-7.6); and was soft (hardness 40-48 and alkalinity 30-35 as mg/l of CaCO_3).

The well water was hard, having a total alkalinity of 331, total hardness of 384, and calcium hardness of 260 (all as mg/l of CaCO_3). Resistivity at 25 C was 1,277 ohms and pH was 7.42. Chemical constituents (mg/l) included ammonia nitrogen, 0.38; nitrite, 0.005; nitrate, 0.05; sulfate, 42.5; orthophosphate, 0.05; total iron, 0.28; manganese, <0.05; sodium, 32.5; calcium, 59.0; magnesium, 18.3; and potassium, 3.7.

Hexagenia nymphs were used as experimental animals, and cultures were maintained in the laboratory according to methods described by Fremling (1967). Relatively large nymphs (20-22 mm) were used in all experiments because they were easy to handle and observe. Last instar nymphs were not knowingly used because physiological stresses involved in transformation to the adult stage are atypical and emergence during the experiments was not desired. Although most test nymphs were H. bilineata from laboratory

cultures, their number was supplemented in all experiments by nymphs of *H. bilineata* and *H. limbata* collected from the Mississippi River. Species collected from the river were not separated because the nymphs were not in their last instar and because undue handling was undesirable. Nymphs collected from the river were placed in laboratory rearing tanks to acclimate for at least 1 wk before they were used in tests.

Nymphs were collected from the rearing tanks by gently sifting mud through a coarse screen; they were then transferred to fresh

water where they acclimated for 6 h before being used in tests. A large syringe, filled with test water from the appropriate vessel, was used to flush the remaining nymphs from their burrows to determine if any were dead. Each nymph was classified as normal, dead, or stressed (as indicated by active swimming, rapid gill movements, or loss of equilibrium). All tests were conducted in a basement laboratory which had no windows. Overhead incandescent lamps provided constant light.

Special glass toxicity test vessels (Fig. 1) were assembled with silicone glue. Each

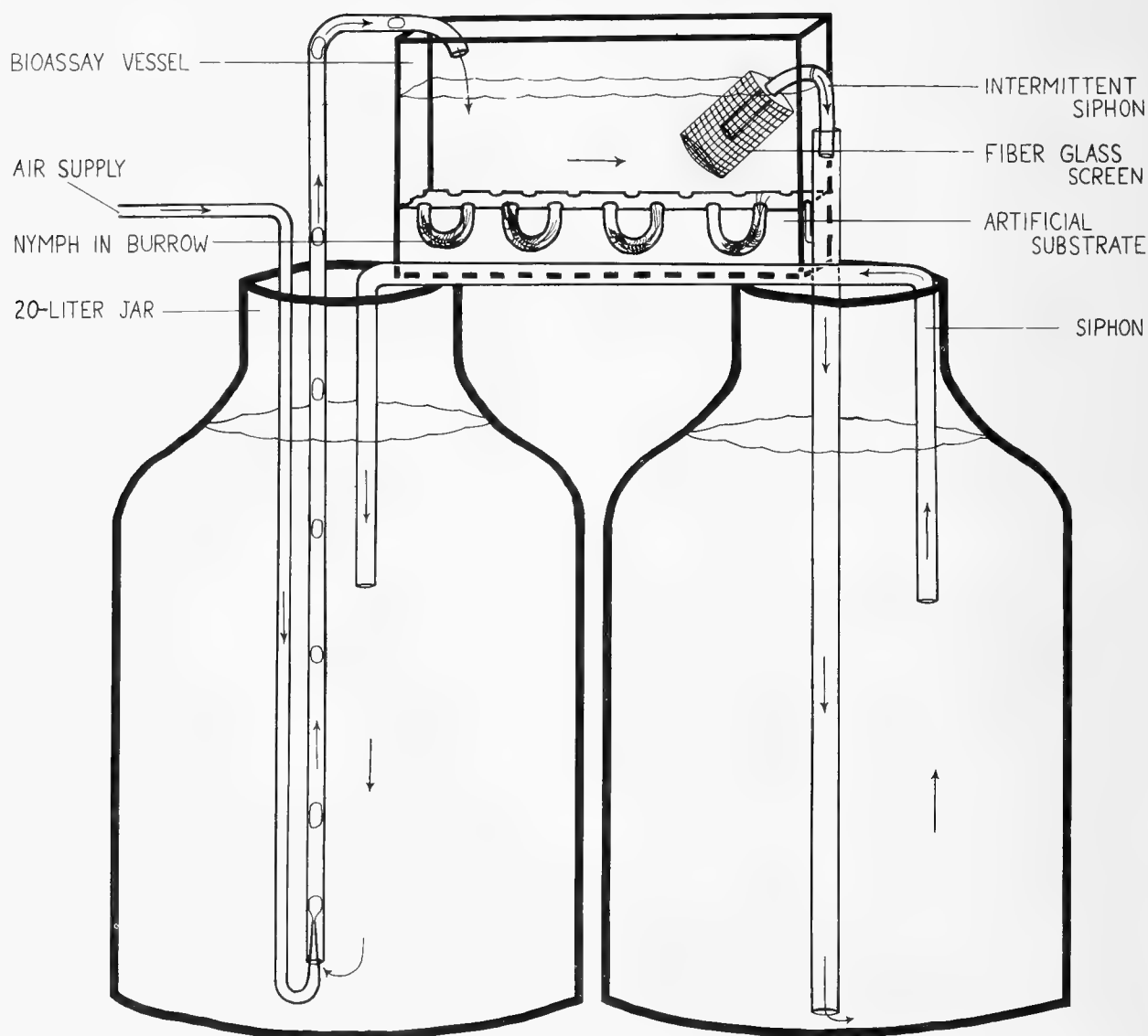


Fig. 1. Recycling bioassay apparatus with a burrow-containing epoxy substrate for use with *Hexagenia* mayfly nymphs and nonvolatile test chemicals.

vessel contained an epoxy resin substrate, 23 cm long, 5 cm wide, and 5 cm deep. Each substrate contained 10 burrows constructed as described by Fremling and Schoening (1973). Constant recirculation of test water through each vessel was assured by pumping water via an air lift (made of 4 and 6 mm I.D. glass tubing) from a 20-liter glass jar through the test vessel to another 20-liter glass jar via an intermittent siphon. A fiber glass screen prevented the escape of test animals. Water was constantly returned by a siphon (10 mm I.D. glass tubing) from the second jar to the first. Recirculation of the test water, which was permissible because TFM is relatively nonvolatile, made it possible to maintain precise control over toxicant concentrations. Accumulation of inhibitory concentrations of degradation and metabolic products was unlikely because of the large volume of water used (38 liters).

Six two-jar units were placed in each of two large water baths so that temperature could be accurately controlled. Each series of six units included one control unit in which no TFM was used and five units which contained various concentrations of TFM.

Stock solutions of field grade TFM (35.7%) and purified TFM (95%) were made by dissolving the chemical in acetone and diluting it

with water. Stock solution was added in equal amounts to both jars of each unit, stirred thoroughly, and allowed to circulate between the jars until mixing was complete. Concentrations of TFM were monitored before nymphs were added and periodically throughout each experiment with a Beckman DB spectrophotometer (Olson and Marking 1973).

Waters of various hardnesses and pH's were used, as described by Marking (1969) and Marking and Dawson (1972), respectively. LC50 values and 95% confidence limits for each test were determined according to methods described by Litchfield and Wilcoxon (1949).

RESULTS

Purified and field grade TFM were toxic to mayfly nymphs at all temperatures tested (17.0 - 26.5 C--see Table 1). The change in toxicity was usually insignificant ($P>0.05$) for single temperature increments; an exception being the increment between 23.5 and 24.4 C in hard water at 24-h exposure. The exception indicates that TFM is more toxic at the higher of the two temperatures. Since the temperature difference is small, however, the data may reflect biological variation among groups of organisms rather than an influence of temperature. Although TFM is toxic in short

Table 1. Toxicity of TFM (based on active ingredient) to *Hexagenia* mayfly nymphs in waters of different hardness and temperature. LC50 values and 95% confidence intervals (in parentheses) are listed as $\mu\text{l/l}$ for 35.7% TFM and as mg/l for 95% TFM.

Hardness (mg/l)	Temp. (°C)	Formulation of TFM (%)	Hours of exposure			
			6	12	24	96
384	17	35.7	--	10.5	6.50	3.90
			--	(8.47-13.0)	(5.23-8.07)	(3.0-5.07)
384	21.8	95	--	--	6.00	4.30
			--	--	(5.18-6.95)	(3.45-5.36)
384	23.5	95	--	10.5	7.00	4.20
			--	(9.38-11.8)	(5.79-8.47)	(3.41-5.17)
384	24.4	95	11.2	6.50	3.50	--
			(10.0-12.5)	(5.21-8.11)	(2.28-5.36)	--
44	18.2	35.7	--	--	4.75	2.50
			--	--	(4.20-5.37)	(1.80-3.46)
44	26.5	35.7	--	4.70	3.50	2.18
			--	(3.96-5.58)	(2.96-4.14)	(1.73-2.74)

exposures (12-h or less), LC50's at these exposures are not much greater than those for 24-h exposures. Comparisons made over a wider temperature range might show greater significance.

Water hardness influenced the toxicity of TFM to Hexagenia nymphs. After 24-h exposures, TFM was considerably more toxic in soft water than in hard water of similar pH (7.1-7.6) and temperature (Table 1). The 24-h LC50's were 6.50 and 4.75 $\mu\text{l/l}$ of TFM in hard and soft water, respectively, at the lower temperatures (17.0 and 18.2 C). At the higher temperatures (24.4 and 26.5 C), TFM was more toxic in soft than in hard water at the 12-h exposure but the difference was nil at 24-h.

The toxicity of TFM to Hexagenia mayfly nymphs was influenced drastically by the pH of water (Table 2). The 24-h LC50 at pH 6.5 (2.50) was significantly greater ($P < 0.05$) than that at pH 7.5 (3.35), and the LC50 at pH 8.5 (18.8) was more than 5 times the value at pH 7.5 (3.35). The toxicity of TFM to the nymphs was lowest at pH 9.5 and the 24-h LC50 was almost 70 times greater than that value at pH 6.5.

Table 2. Toxicity of TFM (35.7%) to Hexagenia mayfly nymphs in soft water (40-48 mg/l total hardness as CaCO_3) at temperatures of 22-23 C and at selected pH values. LC50 values and 95% confidence intervals (in parentheses) are listed as $\mu\text{l/l}$ TFM.

pH	Hours of exposure				
	12	24	48	72	96
6.5	4.00 (3.47-4.61)	2.50 (2.16-2.90)	1.31 (1.04-1.65)	-- --	1.18 (0.91-1.53)
7.5	-- --	3.35 (2.99-3.76)	2.50 (2.17-2.87)	2.00 (1.63-2.46)	-- --
8.5	27.3 (22.4-33.0)	18.8 (16.6-21.4)	13.0 ¹	-- --	5.00 (3.68-6.80)
9.5	270 (221-329)	174 (156-194)	100 (87.2-115)	64.2 (49.5-83.3)	60.0 (45.7-78.8)

¹No confidence interval reported because of insufficient data.

DISCUSSION

TFM is apparently less toxic to some invertebrate animals than it is to lampreys. Experiments by Erkkila (1962) revealed that concentrations of TFM below 20 $\mu\text{l/l}$ caused insignificant mortality to isopods, gammarids, crayfish, dragonflies, water boatmen, and case-building caddisflies; that concentrations below 10 $\mu\text{l/l}$ were harmless to glossiphoniid leeches, stoneflies, bloodworms and snails; and that mortality was significant in Hexagenia at 6 $\mu\text{l/l}$. Smith (1967) showed that mortality of hydras, turbellarians, blackflies, and Hexagenia mayflies was almost complete in the laboratory when these animals were exposed to TFM at 10 $\mu\text{l/l}$ for prolonged periods.

In the present study, TFM was toxic to Hexagenia mayflies in ranges similar to those reported above. Except at pH of 8.5 or over, the 24-h LC50 of TFM was always less than 10 $\mu\text{l/l}$. At low pH in soft water the material was especially toxic. At pH 6.5, for example, the 96-h LC50 was 1.18 (Table 2).

Hexagenia nymphs are less sensitive than larval lampreys to TFM when both species are tested in standard laboratory water. Dawson et al. (in press) found the 24-h LC99 for ammocoetes to be 0.90 at pH 6.5, 3.25 at pH 7.5, and 12.0 at pH 8.5. In the present study, 24-h LC50 values for Hexagenia nymphs were 2.50 at pH 6.5, 3.35 at pH 7.5, and 18.8 at pH 8.5.

In all tests there was a marked tendency for treated nymphs to abandon their burrows for varying lengths of time before they actually succumbed. In nature, this behavior would, on one hand, increase the vulnerability of nymphs to predation; on the other hand, however, free-swimming nymphs might swim, or be swept by the current, out of the zone of lethal TFM concentrations. There is no assurance, however, that nymphs would find suitable substrate in an open lake or that they would recover from the effects of the toxicant.

Hexagenia mayflies are able to recolonize denuded areas by downstream drift of nymphs and by upstream flight of ovipositing adults (Fremling 1973). It is likely that Hexagenia populations killed by TFM applications would become reestablished. Complete reestablishment would probably require a year or more, however.

The artificial substrate apparatus used in this study proved very satisfactory as indicated by the fact that in 10 96-h tests the controls showed no mortality in three tests, 10% in five tests, 20% in one test, and 30% in one test. In the test in which mortality was 30%, two of the dead were nymphs which died during transformation to the subimaginal stage. Nymphs frequently molted to the next nymphal instar in the bioassay vessels.

Artificial substrates such as those used in this study provide semidarkness, thigmotactic surfaces, and seclusion for test nymphs. Consequently, the nymphs swim less and their susceptibility to toxicants is not enhanced by fatigue as it is in standard test vessels.

CONCLUSIONS

1. A recycling toxicity test apparatus with artificial substrates was suitable for tests of TFM against Hexagenia nymphs.
2. Toxicity of TFM to Hexagenia nymphs is relatively independent of temperature.
3. Toxicity of TFM to Hexagenia nymphs is greater in soft than in hard water.
4. Toxicity of TFM to Hexagenia nymphs is much greater at low than at high pH's.
5. Although TFM is more toxic to ammocoetes than to Hexagenia nymphs in soft water, applications of TFM that exceed the minimum effective concentrations for lamprey larvae may kill the nymphs.

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**59. Toxicity and Residue Dynamics of the Lampricide
3-Trifluoromethyl-4-nitrophenol (TFM) in Aquatic
Invertebrates**

By Herman O. Sanders and David F. Walsh



**United States Department of the Interior
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TOXICITY AND RESIDUE DYNAMICS OF THE LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) IN AQUATIC INVERTEBRATES

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ABSTRACT

Six species of aquatic invertebrates including scud, Gammarus pseudo-limnaeus, daphnid, Daphnia magna, crayfish, Orconectes nais, aquatic sowbug, Asellus brevicaudus, damselfly nymph, Ischnura verticalis, and a mayfly nymph, Stenonema sp. were exposed to TFM in toxicity tests in hard water at 21 C.

The 96-h LC50 of field grade TFM (35.7%) was 57.0 mg/l for scud and 110 mg/l for crayfish; purified TFM (95.7%) was twice as toxic. The thirty-day LC50 of field grade TFM was 14 mg/l for scud and 20 mg/l for crayfish. LC50 values are based on whole formulation rather than active ingredient.

Uniformly ^{14}C -ring labeled TFM was employed in the accumulation experiments. All organisms accumulated TFM concentrations within 7 days that were up to 58 times (wet weight of whole organism) the concentration in water. The biological half-life of TFM in scud was 3.5 days. No reproductive impairment occurred in daphnids exposed to 2.4, 4.9, and 10 mg/l field grade TFM for three generations (63 days). Reproduction stopped, however, within the first generation when exposed to 18 mg/l.

INTRODUCTION

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) was registered in 1964 for limited use in tributaries of the Great Lakes for control of the parasitic sea lamprey (Petromyzon marinus). In 1970, however, the registration of TFM was cancelled by the Environmental Protection Agency because of insufficient information on its residues and impact on aquatic organisms other than lamprey. Since cancellation, extensions have been granted to provide time for the Great Lakes Fishery Commission to generate the necessary information for registration.

The physical and chemical properties, efficacy, and toxicity of TFM to aquatic organisms have been compiled in an excellent review by Schnick (1972). This review is extensive and indicates that only a few studies

report on the toxicology of TFM in aquatic invertebrates. Laboratory and field studies have shown that TFM is not acutely toxic to most fish (Applegate and King 1962; Applegate et al. 1961) and invertebrates (Erkkila 1962; Smith 1967) when used at concentrations that kill lamprey larvae (Applegate et al. 1958).

Laboratory studies with TFM and invertebrates have been concerned primarily with acute toxicity. Since accumulation and biological effects of TFM residues in aquatic invertebrates are not understood, we initiated this study to determine toxicity, uptake, dissipation, and residue magnification of TFM in invertebrates. In addition, we evaluated the effects of TFM on reproduction in daphnids, Daphnia magna. The data in this report are intended to assist in the registration of TFM and in establishing criteria for permissible

TFM concentrations in the aquatic environment.

MATERIALS AND METHODS

Test Animals

Test animals consisted of four species of crustaceans and two species of early instar aquatic insects: mature scud (Gammarus pseudolimnaeus Bousfield); early instar and mature daphnids (Daphnia magna Strauss); 14-day-old crayfish (Orconectes nais Faxon); mature aquatic sowbug (Asellus brevicaudus Forbes); damselfly nymph (Ischnura verticalis Say); and mayfly nymph (Stenonema sp.). Daphnids were from laboratory cultures; all other invertebrates were collected from streams and ponds near the Fish-Pesticide Research Laboratory, Columbia, Missouri. Invertebrates collected in the field were placed in acclimation tanks for at least 7 days before testing.

The water used for cultures and all experiments was from a deep well and had the following characteristics: pH 7.2-7.4 and total hardness 270 mg/l as CaCO_3 .

Toxicity Tests

The acute toxicity of field grade TFM (35.7% active ingredient) and purified TFM (95.7% active ingredient) was determined by the standard 96-h static toxicity test (Sanders 1970) and LC50 values were calculated on the basis of the total formulation. Thirty-day flow-through tests with field-grade TFM were conducted using a flow-through diluter after Mount and Brungs (1967). In the flow-through tests, scud were fed coarsely chopped maple and elm leaves and crayfish were fed enriched fish-food pellets. All tests were conducted at $21 \pm 1^\circ\text{C}$.

Toxic effects were measured in terms of the median lethal concentration (LC50), the toxicant concentration in water which is lethal to 50% of the test animals under the test conditions. In flow-through tests, the incipient LC50 or lethal threshold concentration (Sprague 1969) was determined when the asymptote had been reached in the toxicity

curve. This value was determined when the mortality in each aquarium in any 5-day period dropped to 10% of the original number of animals. Toxicity estimates (LC50 values) and corresponding 95% confidence intervals were determined by the Litchfield Wilcoxon method (1949).

Uptake Method

Uptake of TFM from water by the four species of crustaceans and two species of immature insects was studied at concentrations of 0.013, 0.020, 0.026, and 0.510 mg/l.

Uniformly ^{14}C -ring labeled TFM (specific activity 3.66 mCi/mM) was used in the accumulation experiments. A sample of TFM examined by direct probe mass spectrometry contained 0.02% non-volatile ^{14}C -impurities, but no impurities of higher molecular weight were observed (Analyst, D. L. Stalling, Fish-Pesticide Research Laboratory, Columbia, Mo.).

Stock solutions of ^{14}C -TFM were prepared in water and further diluted to desired concentrations in a flow-through system. The water in each aquarium was renewed at a rate of 120 ml/h. The organisms were exposed in two-liter glass aquaria containing one liter of well water. The flow-through system was operated for at least 24 h prior to addition of organisms to allow for concentration equilibrium. The organisms were not fed during the accumulation experiments.

Invertebrate samples were taken in triplicate, weighed, and prepared directly for radiometric analyses by homogenizing the whole organism in a tissue grinder. The homogenate was obtained by adding 6 ml of Triton X-100®; toluene (2:3 v/v) emulsifier to each sample during grinding (Johnson et al. 1971). This homogenate was then transferred to a scintillation vial with three 3 ml washings of a toluene-fluor mixture (5 g of diphenyloxazole (PPO) in 1 liter of toluene). The concentration of TFM in water was monitored radiometrically by taking triplicate 1 ml samples of aquarium water directly into a scintillation vial and then adding 14 ml of Triton/toluene-fluor mixture. The radioactivity

in the tissue and water samples was measured with a Beckman 200-L liquid scintillation spectrometer. Residue values and magnification factors (residue concentration in organism/residue concentration in exposure water) presented in the text and tables were computed on a whole-body, wet weight basis.

Dissipation Method

Dissipation of TFM residues in scud was determined by exposing the organisms to TFM until a plateau concentration was reached. The scud were then transferred to TFM-free flowing water and analyzed periodically to measure decline in whole-body residues.

Reproduction Studies

Reproductive studies with daphnids were conducted in a flow-through system designed for exposing small organisms to constant concentrations of a toxicant over an extended period. Ten first-instar daphnids, up to 24 h old, were placed in duplicate exposure vessels containing 1 liter of water. An aqueous stock solution of field grade TFM was prepared and then further diluted with water to concentrations of 2.4, 4.9, 10, and 18 mg/l. A control was included with each test. Daphnids were fed a suspension of yeast and algae in sufficient amounts to support a stable population. Reproductive success was assessed by counting the offspring produced in each TFM concentration after the parent daphnids had been exposed for 21 days. At the end of 21 days, 10 of the young from each concentration were placed in new media and the 21-day procedure was repeated.

RESULTS

Acute toxicity

Static 96-h toxicity tests indicated that field grade TFM (35.7% AI) and purified TFM (95.7% AI) have relatively low acute toxicities to scud and crayfish (Table 1). The 96-h LC50 value of field grade TFM was 57 mg/l for scud and 110 mg/l for crayfish. The 96-h LC50 value of purified TFM was 22 mg/l for scud and 55 mg/l for crayfish. The toxicity to both animals appears to be related to the level of active ingredient of the compound.

Chronic toxicity

Thirty-day flow-through toxicity tests of field grade TFM with scud resulted in progressively lower LC50 values ranging from 43 mg/l at 5 days to 14 mg/l at 30 days (Table 2). The incipient LC50 was 14 mg/l which was attained in 20 days. The LC50 values for crayfish exposed to field grade TFM declined from greater than 100 mg/l at 1 day to 20 mg/l at 30 days. The incipient LC50 for crayfish was 20 mg/l.

The LC50 values for 4-day exposures of scud to field grade TFM were similar in static and flow-through tests. However, crayfish exposed for 4 days in the flow-through tests were resistant to TFM concentrations twice as high as those in the static test.

Uptake Study

All invertebrates exposed continuously to sublethal concentrations of ^{14}C -TFM accumulated radioactive residues in 7 days that were up to 58 times the concentration in water (Table 3). After an initial rapid uptake, most invertebrates accumulated TFM at a slow rate until a plateau was reached at 7 days. Daphnids, however, accumulated plateau concentrations after a 1-day exposure.

Accumulation of TFM residues by invertebrates appears dependent upon the concentration in water, but magnification factors are relatively independent of these concentrations. After a 7-day exposure to 0.013 mg/l, scud concentrated TFM 58 times (0.754 mg/kg) the level in water. When scud were exposed to 0.510 mg/l, they accumulated total body concentrations 56 times (28.6 mg/kg) that of water.

A comparison of the results from our accumulation experiments indicates a significant difference in the rate of uptake and residue magnification of TFM by the various organisms. TFM uptake from water by aquatic insects was relatively low when compared to uptake by crustacea. However, of all the organisms investigated, crayfish accumulated the least TFM concentrations from water.

Table 1. Toxicity¹ of field grade TFM and purified TFM to scud and crayfish

Organism	LC50 values ² (mg/l) and 95% confidence intervals at --			
	24 h		96 h	
	Field grade	Purified	Field grade	Purified
Scud (mature)				
<u>Gammarus pseudolimnaeus</u> ..	100 (83-130)	28 (23-34)	57 (47-69)	22 (16-31)
Crayfish (14 days old)				
<u>Orconectes nais</u>	130 (115-150)	60 (45-80)	110 (90-125)	55 (48-70)

¹Static toxicity test. Hard water (pH, 7.2-7.4, total hardness 270 mg/l as CaCO₃) at 21° C.

²Values based on whole formulation rather than active ingredient.

Table 2. Flow-through toxicity tests¹ of field grade TFM against scud and crayfish

Organism	LC50 values ² (mg/l) and 95% confidence intervals at --					
	1 day	4 days	10 days	15 days	20 days	30 days
Scud (mature)						
<u>Gammarus pseudolimnaeus</u> .	>100	43 (29-57)	30 (19-48)	28 (17-44)	14 (11-23)	14 (11-23)
Crayfish (14 days old)						
<u>Orconectes nais</u>	>100	34 (21-47)	20 (12-32)	20 (12-32)	20 (12-32)	20 (12-32)

¹Hard water (pH, 7.2-7.4; total hardness 270 mg/l as CaCO₃) at 21 C.

²Values based on whole formulation rather than active ingredient.

Table 3. Uptake and magnification of ^{14}C -TFM by six aquatic invertebrates

Organism	Organisms ¹ per sample	Water ² concentration (mg/l)	Whole body residues (mg/kg) and magnification factor ³				
			1 day	4 days	7 days	14 days	21 days
Scud (mature)..... <u>Gammarus pseudolimnaeus</u>	3	0.013	0.390 (30)	0.728 (56)	0.754 (58)	0.754 (58)	--
Scud (mature)..... <u>Gammarus pseudolimnaeus</u>	3	0.026	0.286 (11)	0.702 (27)	1.35 (51)	1.33 (51)	1.35 (52)
Scud (mature)..... <u>Gammarus pseudolimnaeus</u>	3	0.510	8.45 (16)	22.1 (43)	28.6 (56)	45.7 (89)	--
Waterflea (mature)..... <u>Daphnia magna</u>	60	0.026	0.120 (5)	0.136 (5)	--	--	--
Mayfly (early instar)..... <u>Stenonema sp.</u>	6	0.026	0.0360 (1.3)	0.056 (2.2)	0.110 (4.4)	--	--
Damselfly (early instar)... <u>Ischnura verticalis</u>	3	0.510	0.15 (0)	0.63 (1.2)	0.49 (1)	--	--
Crayfish (immature, 21 days)..... <u>Orconectes nais</u>	2	0.026	0.0360 (1.3)	0.048 (1.8)	0.052 (2)	0.068 (2.6)	0.068 (2.6)
Sowbug (mature)..... <u>Asellus brevicaudus</u>	4	0.020	0.28 (14)	0.74 (37)	--	--	--

¹Samples were taken in triplicate.²Hard water (pH 7.2 and total hardness 270 mg/l as CaCO_3) at 21 C.³Concentration in organism (wet weight)/concentration in water.

Dissipation study

The dissipation of TFM residues by scud was determined by exposing them to 0.026 mg/l of ^{14}C -TFM for 7 days. This exposure was sufficient to induce a residue plateau. Once this plateau was reached, the scuds were transferred to TFM-free flowing water. The time required for 50% elimination of TFM by scud was 3.5 days. At 14 days, 98% of the radioactive residues had been lost. Analytical techniques to determine TFM degradation products in invertebrates are not well defined but it is assumed that the loss of radioactivity was due to excretion of TFM and/or metabolites of TFM.

Reproduction study

Continuous exposure of daphnids for three generations (63 days) to 2.4, 4.9, and 10 mg/l of field grade TFM (35.7% active ingredient) did not significantly impair reproduction when compared with controls. However, daphnids exposed at 18 mg/l of field grade TFM formed ephippial eggs (fertilized eggs) and reproduction stopped within the first generation (21 days).

DISCUSSION

Of the various chemicals toxic to sea lamprey, TFM is considered the most desirable for use in Great Lakes tributaries

because of its effectiveness as a lamprey larvicide and its safety to resident fish populations. Concentrations of 2-4 mg/l of TFM required to kill lamprey larvae in streams (Applegate et al. 1958) are not toxic to most aquatic invertebrates during acute exposures (Smith 1967; Erkkila 1962). Observations in the field have also shown that fish and reptiles (Applegate et al. 1961), and amphibians (Johnson 1959) are not affected by these TFM treatments.

Comparable data on the toxicities of TFM to aquatic invertebrates are limited to a few animals because many investigators have not included information on water quality or grade of TFM used in their experiments. Our LC50 values for scud and crayfish are in agreement with those of Erkkila (1962) and Smith (1967) who reported 24-h LC50 values of greater than 20 mg/l for both invertebrates. Applegate et al. (1958) found that the toxic effects of mononitrophenols on fish were considerably less under conditions that simulated treatment of an actual stream than effects which were observed under static conditions. Similar observations were noted in our studies, in which TFM was twice as toxic to crayfish in static toxicity tests than in flow-through tests.

Toxic chemicals introduced into the aquatic environment are often below levels acutely toxic to invertebrates. These sublethal concentrations, however, may impair successful growth, molting, and reproduction in invertebrate populations. Therefore, populations of fishes may be threatened because of loss of the fish-food organisms. The results from our reproductive toxicity tests indicate no reproductive impairment in *D. magna* when they were exposed continuously for three successive generations (63 days) to 10 mg/l of field grade TFM. Because of material cost, the continuous application of TFM in the aquatic environment never exceeds 24 h (Applegate and King 1962). Therefore, it seems highly improbable that daphnids would ever be exposed to concentrations greater than 10 mg/l, especially for 63 consecutive days.

Our knowledge of the metabolic fate of TFM in fish and invertebrates is incomplete. Lech (1971) found that TFM was degraded in rats to

3-trifluoromethyl-4-aminophenol (RTFM). He also found that TFM and RTFM were excreted in the urine as polar derivatives, some of which appear to be glucuronides. Although we only determined the loss of ^{14}C -TFM residues in scud, our results suggest that the dissipation of these residues was rapid. Therefore, significant TFM concentrations would not be expected to accumulate in top level consumers. Further studies, however, are needed to determine residue data from various components of simulated or natural intact food chains. The method of application, proper formulation, water quality characteristics, and species of animals in the area of TFM application are important factors to be considered in minimizing the hazard of TFM to aquatic animals.

SUMMARY

The acute toxicities of field grade TFM (35.7%) and purified TFM (95.7%) were determined for scud and crayfish in well water (pH 7.2 and total hardness 270 mg/l) at 21 C. The 96-h LC50 of field grade TFM was 57 mg/l for scud and 110 mg/l for crayfish; purified TFM was twice as toxic. The 30-day LC50 of field grade TFM was 14.3 mg/l for scud and 20.1 mg/l for crayfish.

Six species of aquatic invertebrates exposed to ^{14}C -TFM accumulated residues up to 58 times (wet weight) the concentration in water.

The amount of TFM accumulated by scud at equilibrium (7 days) was proportional to the concentration in water. However, magnification factors were relatively independent of water concentrations. When scud were transferred to TFM-free flowing water after 7 days of exposure to 0.026 mg/l of TFM, the residues decreased at 14 days from 0.754 mg/kg to 0.03 mg/kg.

Concentrations of 2.4, 4.9, and 10 mg/l of field grade TFM did not significantly impair reproduction in daphnids after 63 days of exposure. Total production of young was inhibited in 21 days at a concentration of 18 mg/l.

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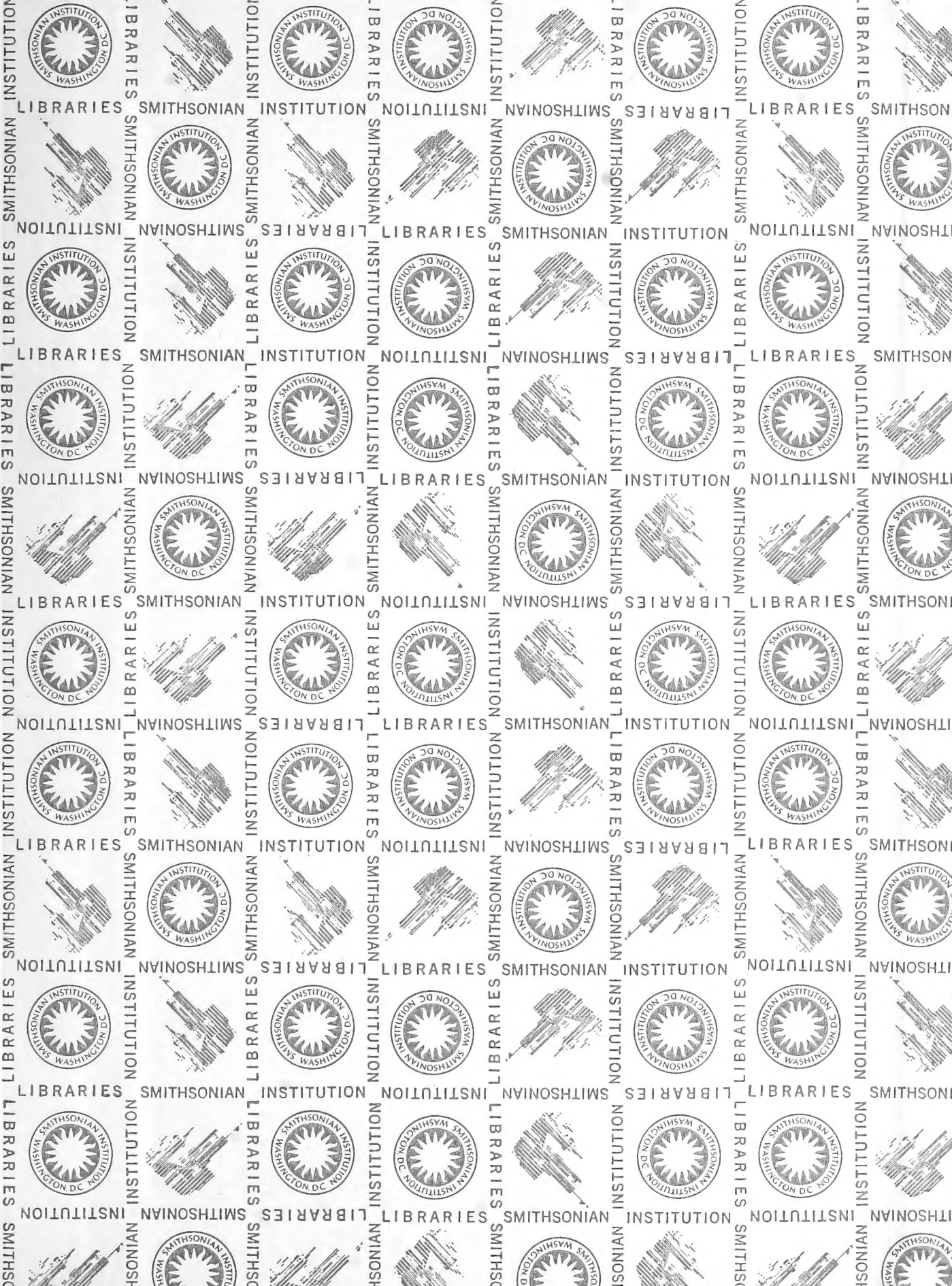


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